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Contribution à la robustesse des traitements fongiques et enzymatiques de contaminants organiques persistants dans des matrices environnementales complexes

Thèse de doctorat

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RÉSUMÉ

Les effluents industriels et domestiques représentent les principales sources de contamination des milieux aquatiques par une variété de substances organiques persistantes. L'élimination de ces substances dans les eaux usées relève d'un défi technologique qui nécessite l'adaptation ou l'amélioration des systèmes de traitement conventionnels des eaux actuelles. Les approches de mycoremédiation exploitant les champignons responsables de la pourriture blanche du bois (WRFs) et celles de biocatalyse enzymatique utilisant des enzymes fongiques isolées constituent des méthodes versatiles et durables pour le traitement de ces matrices complexes. Malgré leur efficacité, ces techniques souffrent d'un manque de robustesse qui freine leur usage à l'échelle industrielle. En effet, la majorité des études concernant leur utilisation ont été réalisées à l'échelle du laboratoire dans des conditions optimales contrôlées et peu représentatives des matrices complexes ainsi que des concentrations ciblées. L'exploitation de ces technologies présente un défi pour la communauté scientifique. Ce projet de recherche vise à mettre en place, et à évaluer des stratégies contribuant à l'amélioration de la robustesse des traitements fongiques et enzymatiques.

Les travaux développés dans ce document ont porté sur deux aspects : 1 – la mise en place de stratégies de sélection de souches fongiques dans un effluent industriel de bioraffinerie riche en composés phénoliques. 2 – l'évaluation d'une méthode pour la stabilisation d'un extrait brut de laccase pour l'élimination de contaminants dans les effluents municipaux.

Le choix était basé sur l'aptitude de sept souches de champignons à éliminer les phénols totaux de l'effluent, ainsi que sur leur capacité à sécréter la laccase. Les deux stratégies employées (l'une utilisant du glucose et l'autre des copeaux de bois comme cosubstrat pour la croissance des souches) ont permis d'identifier *P. dryinus* et *T. hirsuta.* Ces deux souches ont permis d'éliminer les phénols totaux de l'effluent à des taux supérieurs à 94 %, avec les deux stratégies testées. De plus, sur les sept souches étudiées, seules *P. dryinus* et *T. hirsuta* ont produit la laccase. Ces résultats établissent la possibilité d'exploiter *P. dryinus* et *T. hirsuta* pour le prétraitement de l'effluent issu de la valorisation industrielle des bois traités en fin de vie. Cette conclusion ouvre la voie au développement de procédés de mycoremédiation durables. Cette expérience suggère également que la valorisation des déchets lignocellulosiques peut se révéler

une alternative économique pour la mise en place d'une telle approche de traitement. En effet, l'utilisation des copeaux de bois comme cosubstrat a démontré une meilleure efficacité en ce qui concerne la production de la laccase (une enzyme au fort potentiel biotechnologique). Cependant, une pareille approche nécessite encore d'être optimisée.

L'insolubilisation reste un des moyens les plus efficaces pour la stabilisation et l'utilisation des enzymes isolées dans des bioprocédés opérant en continu. La laccase brute insolubilisée sous forme de CLEAs (*cross-linked enzyme agregates*) demeure une méthode prometteuse pour des applications environnementales. Pourtant, la transition de cette technologie vers des applications industrielles est limitée par sa faible résistance mécanique. Cette thèse a permis de confirmer, la possibilité d'employer la méthode EPES (*enzyme polymer engineered structure*) pour la stabilisation d'un extrait brut de laccase de *T. hirsuta*. La caractérisation des EPES-CLEAs formés a démontré une nette augmentation de la stabilité des CLEAs dans différentes conditions dénaturantes. De plus, les résultats ont démontré une meilleure recyclabilité et une préservation de l'efficacité du biocatalyseur dans un effluent municipal.

En somme, ces travaux apportent de nouvelles connaissances sur l'amélioration des procédés de mycoremédiation et de biocatalyse enzymatique des contaminants organiques persistants dans des matrices complexes. Ils démontrent en particulier l'intérêt de caractériser ces technologies novatrices dans les conditions typiques de l'environnement afin d'augmenter la robustesse des procédés.

Mots clés : Contaminants organiques persistants, Eaux usées, Mycoremédiation, Enzymes immobilisées, Agrégats d'enzyme réticulée, Laccase, Stabilisation.

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ABTS : 2,2'-azino-bis-(3-éthylbenzothiazoline-6-sulfonic acid

AINS : anti-inflammatoires non stéroïdiens

APTES : 3-aminopropyltriethoxysilane

CHI : chitosane

CLEAs : *cross-linked enzymes aggregates*

CLECs : *cross-linked crystallized enzymes*

CLEs : *cross-linked enzymes*

CLSD : *cross-linked spray dried enzymes*

DCO : demande chimique en oxygène

EDAC : éthyl-3-(3-diméthylamino-isopropyl) carbodiimide

EPES : enzyme polymer engineered structure

GLU : glutaraldéhyde

HAPs : hydrocarbures aromatiques polycycliques

HOBt : hydroxybenzotriazole

PPhAs : produits pharmaceutiques actifs

SA : sulfate d'ammonium

SENs : *single enzyme nanoparticules*

STEPs : stations d'épuration

TrOCs : contaminants organiques traces

WRFs : *white rot fungus*

Chapitre 1 - Introduction générale

1.1 Mise en contexte

Les effluents issus des activités anthropiques (effluents municipaux et industriels) demeurent la principale voie d'entrée de contaminants organiques persistants (ex. : produits pharmaceutiques actifs (PPhAs), hydrocarbures aromatiques polycycliques (HAPs), chlorophénols) dans l'environnement [1]. L'impact de ces substances sur la santé humaine et les écosystèmes n'est plus matière à discussion [2,3]. L'élimination de ces contaminants des eaux usées représente un défi technologique, avec un réel besoin de moderniser les dispositifs de traitement conventionnels actuels. Plusieurs techniques ont été proposées (ex. : les techniques d'oxydation avancée, les méthodes physiques de filtration, les procédés de bioremédiation) [4–6]. Pour des applications à l'échelle industrielle, la démarche de bioremédiation reste la mieux adaptée, car plus économique et plus respectueuse de l'environnement [7,8].

La bioremédiation consiste à tirer profit des capacités métaboliques des microorganismes pour la dépollution des milieux contaminés. Le principe même des stations d'épuration (STEPs) actuelles est basé sur cette technique. Les STEPs utilisent des consortiums microbiens pour une approche spécifique de dépollution (ex. : élimination de la matière organique). Ces ouvrages sont conçus pour traiter les eaux usées selon les normes en vigueur. Parmi les contaminants organiques d'intérêt environnemental présents à l'état de traces (TrOCs) dans les eaux usées figurent les PPhAs [9]. En absence de réglementation, et face à la diversité et à l'impact de ces contaminants dans l'environnement, la recherche de procédés de traitement alternatifs non spécifiques s'avère nécessaire. Parmi les voies de bioremédiation, la mycoremédiation à l'aide de champignons responsables de la pourriture blanche du bois *ou white rot fungus* (WRFs), apparaît prometteuse [10]. Les WRFs offrent une approche de traitement versatile. À travers leurs mécanismes enzymatiques uniques et leurs potentiels de biosorption, ces organismes ont démontré une grande efficacité à dégrader une multitude de contaminants organiques persistants dans diverses matrices (liquides, solides) [11,12].

La capacité des WRFs à éliminer les contaminants organiques dans l'eau a été établie depuis plusieurs années à l'échelle du laboratoire [10,13]. Des eaux usées synthétiques stérilisées et enrichies en substrats pour la croissance des champignons sont souvent utilisées. De plus, les

contaminants étudiés proviennent en majorité de solutions standards ajoutées au milieu de réactions à des concentrations non représentatives des conditions environnementales. Ces pratiques coûteuses restent incompatibles avec l'application du procédé à grande échelle dans les conditions environnementales réelles. Malgré les conditions contrôlées, les mécanismes d'élimination des contaminants par les WRFs restent complexes et demandent également à être élucidés [14]. Les WRFs ont déjà été expérimentés dans des applications de traitement en continu en condition non stérile. La difficulté de mise en œuvre de cette approche réside dans la compétition entre les WRFs utilisés et les microorganismes indigènes (bactéries et autres champignons) présents dans l'effluent [15]. L'immobilisation des WRFs sur des matériaux lignocellulosiques (ex. : copeaux de bois) présente un double avantage. Cette approche contribue à réduire la viscosité du milieu, causée par la production de biomasse, et permet de tirer avantage du support comme cosubstrat abordable pour la croissance des WRFs [16]. Toutefois, la lenteur du traitement (plusieurs jours) demeure une des principales restrictions à l'application de ce traitement à l'échelle industrielle [4,17,18]. Compte tenu de ces limitations, les applications de niche telles que le prétraitement d'effluents particuliers tels que les eaux usées des moulins à huile d'olive [19] ou de bioraffineries [20] avant leur rejet dans le réseau d'assainissement ou leur réutilisation apparaissent comme l'une des meilleures stratégies.

Depuis quelques années, la durabilité régit les modes d'exploitation industrielle [21]. Le gouvernement du Québec a pris des mesures dissuasives dans le but d'encourager la valorisation et limiter l'enfouissement des déchets de bois traité. Le Canada fait partie des 3 plus grands pays producteurs de bois traité au monde avec une production maintenue à près de 3,5 millions de mètres cubes ces vingt dernières années. La majorité du bois traité produit (90 %) est utilisé par le pays [22]. En fin de vie, ces rebuts sont généralement incinérés ou enfouis avec des risques de contamination de l'environnement. Ces modes de gestion ne s'intègrent pas dans une démarche de développement durable. Une des principales stratégies de valorisation reste l'exploitation industrielle pour la production de biocarburant [23]. La bioraffinerie demeure une approche durable pour la valorisation des déchets lignocellulosiques [24]. Cependant, la revalorisation du bois traité génère des eaux usées composées d'un éventail de contaminants organiques persistants provenant des agents de préservation. L'intérêt pour la mycoremédiation de ce type d'effluent industriel existe [20].

Toutefois, l'efficacité des traitements de mycoremédiation dépend du type de WRFs et des conditions opératoires. De ce fait, une sélection préalable des champignons dans les conditions représentatives de l'application ciblée apparaît déterminante, afin d'assurer la robustesse du traitement.

Une alternative pour pallier les limitations des traitements fongiques consiste à isoler et à utiliser les enzymes extracellulaires des WRFs [4]. Les enzymes fongiques d'intérêt biotechnologique comme les laccases et les peroxydases ont permis d'éliminer une variété de TrOCs dans l'eau [25]. Parmi ces enzymes, cette thèse s'intéresse en particulier à la laccase. Cette enzyme au potentiel versatile apparaît plus avantageuse pour les applications environnementales, car elle n'a besoin que de l'oxygène moléculaire comme cofacteur pour oxyder une large gamme de substrats [26].

Cependant, les laccases libres existent sous forme soluble dans l'eau, et leur utilisation peut s'avérée coûteuse, car, limitée par un manque de recyclabilité et de stabilité vis-à-vis des conditions opératoires. Les pratiques physico-chimiques d'immobilisation des enzymes utilisant des stratégies avec ou sans supports apparaissent efficaces pour assurer la stabilité et la récupération des enzymes du milieu réactionnel [27,28]. Pour des applications environnementales comme le traitement des eaux usées, le choix de la méthode se porte sur des approches relativement peu coûteuses et respectueuses de l'environnement. Les stratégies d'immobilisation sans support (ou insolubilisation), en particulier, la technique des agrégats d'enzymes réticulés (CLEAs) démontrent le plus d'avantages. La méthode CLEA est simple à produire, elle offre une meilleure activité volumétrique et ne requiert pas d'enzymes purifiées [29,30]. La technologie CLEA a permis d'améliorer la stabilité des laccases (lac-CLEAs) face à des conditions dénaturantes telles que les variations de pH et de température, ainsi que la présence de sels et de solvants organiques [31,32]. L'efficacité des lac-CLEAs, pour l'élimination des TrOCs dans l'eau a également été démontrée [33,34]. Par exemple, un extrait brut de laccase de *C. polyzona* insolubilisé sous forme de CLEAs a permis d'éliminer 100 % du Triclosan en solution après 6 h de traitement [35].

Malgré leurs potentiels biotechnologiques, la faible résistance mécanique, et le manque de caractérisation des lac-CLEAs, dans des conditions environnementales réelles crée un fossé entre leur utilisation à l'échelle du laboratoire et leur exploitation à l'échelle industrielle. Pour

être appliquée avec succès dans des conditions dénaturantes comme celles rencontrées dans des matrices complexes, la robustesse des CLEAs doit être améliorée [36]. La souplesse de la méthode la rend compatible avec d'autres techniques existantes. L'encapsulation (E-CLEAs) [34], la fixation sur nanoparticules magnétiques (M-CLEAs) [37] et le greffage de structure polymérique ou *enzyme polymer engineered structure* (EPES) [38], ont été employés pour améliorer la stabilité, et la recyclabilité des lac-CLEAs.

Ces méthodes conduisent à l'augmentation ou à la diminution de la taille des CLEAs, ce qui engendre un compromis entre l'efficacité catalytique du biocatalyseur et leur recyclabilité. En effet, comparées aux particules nanométriques, les particules plus grosses (supérieure à quelques micromètres) apparaissent plus facilement recyclables avec des procédés simples conventionnels. La méthode par encapsulation souffre du risque de fuite du biocatalyseur au travers de la matrice poreuse [39]. L'approche de stabilisation des CLEAs par la méthode EPES semble mieux adaptée, car elle résulte de la fixation par liaisons chimiques du réseau polymérique. Cette technique prometteuse a été proposée il y a quelques années pour la stabilisation de la laccase commerciale de *T. versicolor* [38]. Depuis lors, aucune étude ne s'est intéressée à cette approche pour les applications de bioremédiation environnementale.

1.2 Problématique et questions de recherche

Les systèmes enzymatiques fongiques offrent un potentiel biotechnologique remarquable qui soutient l'aptitude des WRFs pour la bioremédiation des contaminants organiques persistants dans les eaux usées. Toutefois, la mise en place de ce traitement dépend du type d'eau usée, de l'utilisation finale des eaux usées traitées et du coût du traitement.

Les effluents contenant des polluants concentrés en composés chlorés et phénoliques tels que les effluents de l'industrie de blanchiment des pâtes et papiers posent un problème pour les procédés classiques d'épuration des eaux usées. Les WRFs sont connus pour survivre à ces conditions et dégrader les polluants dans ce type de matrice. Ces dernières pourraient ainsi représenter une bonne alternative pour le traitement de ces eaux usées. Cependant, les travaux de recherches actuelles présentent plusieurs lacunes qui constituent un frein à l'exploitation des WRFs dans les situations réelles d'opération. Les traitements fongiques dépendent essentiellement des souches de WRFs utilisées et des conditions opératoires. Le transfert de cette technologie vers des applications environnementales nécessite l'utilisation de matrices

réelles non enrichies et non stériles, afin d'augmenter la robustesse des systèmes. Cela passe également par la sélection préalable des souches fongiques dans des milieux représentatives de l'application ciblée. Cette thèse s'inscrit dans cette démarche et tente de répondre à la question suivante :

- Le prétraitement fongique par l'exploitation des WRFs est-il applicable à un effluent industriel issu de la valorisation du bois traité en fin de vie ? Quelle stratégie de culture apparaît la mieux adaptée pour la sélection des WRFs dans l'effluent étudié ?

Les eaux usées municipales contiennent généralement un mélange d'un large éventail de TrOCs. Les bactéries apparaissent moins polyvalentes pour le traitement de combinaisons de TrOCs. Par exemple, les STEPs de boues activées conventionnelles ne sont pas capables de dégrader la plupart des PPhAs dans les eaux usées municipales. Les WRFs offrent une approche de traitement plus polyvalente. Toutefois, malgré le potentiel de bioremédiation des WRFs et les études intéressantes publiées, la mycoremédiation des eaux usées par les WRFs n'est pas appliquée à l'échelle industrielle, car limitée par des contraintes économiques, et la durée excessive des traitements. L'isolation des enzymes fongiques extracellulaires d'intérêt biotechnologique comme la laccase insolubilisée sous forme de CLEAs représente une technique intéressante pour pallier ces limitations. Cependant, des limitations pratiquent telles que le manque de stabilité et les difficultés de recyclage, limitent encore l'utilisation à l'échelle industrielle des CLEAs. La nécessité de renforcer la robustesse de cette technologie est réelle. Le choix de cette étude visant la stabilisation des lac-CLEAs porte sur la méthode de greffage par liaison chimique d'un réseau de polymères ou *enzyme polymer engineered structure* (EPES). Cette méthode a permis de stabiliser une laccase commerciale de *T. versicolor* insolubilisée sous forme de CLEAs et caractérisé dans des conditions expérimentales contrôlées.

- Dans le but d'appliquer les lac-CLEAs, pour l'élimination des TrOCs dans les eaux usées municipales, cette thèse cherche à déterminer si la méthode EPES permet de stabiliser un extrait brut de laccase insolubilisée sous forme de CLEAs, et quel est l'impact de cette méthode sur les propriétés des lac-CLEAs formés ?

1.3 Objectifs du projet

De manière générale, cette thèse vise d'une part, à mettre en place une stratégie de sélection de souches fongiques en vue du prétraitement d'un effluent industriel issu de la valorisation du bois

traité, et d'autre part, à évaluer la méthode EPES pour la stabilisation d'un extrait brut de laccase destinée à des applications biotechnologiques de traitement des TrOCs eaux usées.

Les objectifs spécifiques suivants ont été définis :

- Déterminer l'aptitude d'une collection de champignons pour le prétraitement d'un émissaire industriel issu de la valorisation du bois traité.
- Démontrer la possibilité de préparer des EPES-CLEAs robustes à partir d'un extrait brut de laccase fongique.
- Caractériser les EPES-CLEAs formés dans une matrice simple (solution tampon) et évaluer leur stabilité dans une matrice complexe (effluent de STEP).
- Utiliser et évaluer l'efficacité des EPES-CLEAs pour le traitement en batch de modèle de PPhAs dans l'eau.
- Estimer les coûts de production des EPES-CLEAs formés.

1.4 Structure de la thèse

À la suite du **chapitre 1** (introduction générale), ce document présente dans le **chapitre 2** un état de l'art succinct qui justifie l'utilisation et fait ressortir les limitations des traitements fongiques et enzymatiques pour l'élimination des contaminants organiques persistants dans l'eau.

Le **chapitre 3** consiste à évaluer deux stratégies de sélection pour la sélection de champignons en vue du prétraitement d'un effluent industriel en condition non stérile.

Le **chapitre 4** comporte un résumé sur les lac-CLEAs, et souligne la nécessité d'améliorer leur potentiel d'application à l'échelle industrielle (ex. : recyclabilité, stabilité).

Le **chapitre 5** propose l'utilisation de la méthode EPES pour la stabilisation d'un extrait brut de laccase sous forme d'EPES-CLEAs et leur application pour l'élimination de modèle de PPhAs dans l'eau.

Le chapitre 6 présente une estimation préliminaire des coûts de production des EPES-CLEAs.

Finalement, le **chapitre 7** fait état des conclusions et des perspectives.

Chapitre 2 - État de l'art

Les contaminants organiques persistants représentent un groupe diversifié de composés chimiques, qui présentent les quatre propriétés suivantes : 1 – ils sont toxiques pour l'environnement et la santé humaine. 2 – ils demeurent persistants dans l'environnement. 3 – ils sont bioaccumulables dans les tissus vivants. 4 – ils peuvent être transportés sur de longues distances [1,40]. Leur présence ubiquiste dans l'environnement particulièrement dans l'eau, constitue un défi technique, sociopolitique et écologique. Leur élimination nécessite le développement, l'évaluation et la mise en œuvre de technologies durables de traitement adapté. Ce chapitre expose les approches de mycoremédiation et de biocatalyse enzymatique de traitement pour l'élimination de ces contaminants dans l'eau.

2.1 Problématique des contaminants organiques d'intérêt dans l'environnement

L'industrialisation et l'urbanisation de nos sociétés entraînent la pollution de l'environnement par un cocktail de contaminants de sources variées. Ces dernières proviennent principalement des rejets directs ou après traitement d'effluents industriels ou domestiques, des contaminations diffuses d'origine agricole ou des ruissellements urbains et retombés atmosphériques [1,41]. Ce document aborde la problématique des agents organiques de préservation du bois traité dans les eaux usées de bioraffinerie issue de la valorisation du bois traité et celle des PPhAs dans les effluents municipaux.

L'utilisation du bois pour les usages domestiques et industriels nécessite un traitement afin de prolonger leur durée de vie contre l'altération atmosphérique et la détérioration due aux microorganismes et assurer la longévité des installations. Le Canada fait partie des trois plus grands pays producteurs de bois traités au monde, avec seulement 10 % exporté [22]. Différents produits chimiques organiques et inorganiques sont utilisés comme agents de préservation. Les agents organiques de préservation du bois comprennent la créosote et le pentachlorophénol (PCP)). Ces composés sont utilisés de manière intensive par les industries de production de travers de chemins de fer et la fabrication de poteaux électriques [23].

La créosote est un distillat de goudron de houille composée majoritairement (85 %) d'hydrocarbures aromatiques polycycliques (HAPs) [23]. Les HAPs sont des substances

chimiques formées d'au moins deux anneaux aromatiques d'atomes de carbone et d'hydrogène. La principale source des HAPs est la combustion ou la pyrolyse de matières organiques telles que le charbon, le pétrole et le bois. Ils sont bioaccumulables et modérément persistants dans l'environnement [42]. Les produits traités à la créosote restent l'une des principales sources de HAPs dans l'eau et le sol avec des quantités annuelles qui peuvent atteindre jusqu'à 2 000 tonnes [43]. La créosote n'est plus utilisée dans le traitement des poteaux des lignes électriques au Canada depuis près de cinquante ans [43]. Ces derniers sont principalement traités avec le PCP. Le PCP est un pesticide organochloré synthétisé par l'homme. C'est un composé organique stable et difficilement biodégradable [44]. Le PCP est commercialisé avec une pureté inférieure à 90 %. Les autres composantes représentent principalement des phénols plus faiblement chlorés tels que les tri et les tétrachlorophénols, les chlorobenzènes, les dioxines et les furanes à l'état de trace [45].

En fin de vie utile, l'enfouissement et l'incinération demeurent les principaux modes de gestions du bois traité. Ces pratiques représentent un risque de dispersion des agents de préservation dans l'environnement. Le gouvernement du Québec a pris des mesures dissuasives concernant l'enfouissement et l'incinération des déchets solides dont le bois traité. Des mesures telles que l'augmentation des frais d'enfouissement afin encourager les industries à adopter des modes de gestion durable basés sur le recyclage et la valorisation des déchets de bois traités [46]. Le compostage, la production de bioéthanol sont des exemples de valorisation envisageables [23]. Cependant, une détoxification préalable des déchets de bois traités est nécessaire du fait de la toxicité des agents de préservation du bois. En effet, ces substances peuvent inhiber l'activité des levures durant le processus de fermentation qui conduit à la production de biocarburant [47]. Les HAPs et les phénols chlorés sont nocifs pour la santé humaine et l'environnement. Certains sont répertoriés par l'Agence de protection de l'environnement des États-Unis (USA-EPA) comme contaminants environnementaux prioritaires, car elles peuvent causer de graves problèmes toxicologiques. La plupart de ces contaminants sont connus pour s'accumuler dans le foie, les reins, les tissus adipeux… etc., leurs effets cancérigènes et mutagènes sont également bien établis [3,48].

Ces deux dernières décennies, la communauté scientifique s'est intéressée à une variété de contaminants organiques persistants présents à l'état de trace (TrOCs) dans l'environnement [49]. Les TrOCs proviennent essentiellement des activités industrielles, domestiques et

agricoles [50]. Les effluents des STEPs figurent parmi les principales sources de TrOCs dans l'environnement, en particulier dans les milieux aquatiques, car les ouvrages de traitement ne sont pas conçus pour éliminer ces contaminants [51]. Les TrOCs comprennent entre autres : les PPhAs (humains et vétérinaires), les produits d'hygiènes et de soins personnels, les nanoparticules, les microplastiques, etc. [1,52].

La figure 2-1 illustre l'attention accordée à ces contaminants depuis l'an 2000. La croissance annuelle exponentielle du nombre de publications sur les 19 dernières années témoigne de l'importance du sujet (Figure 2-1). Cet intérêt est soutenu par les progrès de chimie analytique qui a permis de révéler la présence des TrOCs dans l'environnement, ainsi que la compréhension graduelle des effets néfastes de ces substances sur l'environnement et la santé [1,53].

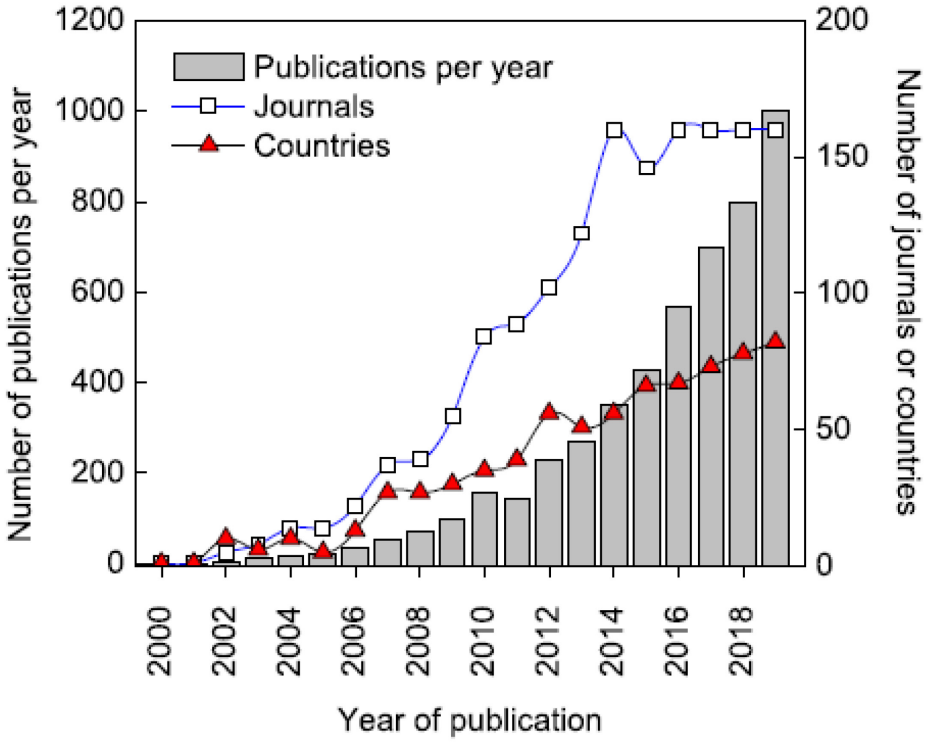

Figure 2-1. Nombre de publications par an entre 2000 et 2019 sur les contaminants émergents. Analyse bibliométrique réalisée à partir de la base de données Scopus (mots clés utilisés : *emerging contaminants, contaminants of emerging concern, pollutants of emerging concern, emerging organic contaminanats, emerging organic pollutants, emerging environmental concern contaminants, emerging environemental concern pollutants* (extrait de [49]).

Parmi les TrOCs d'intérêt environnemental, les PPhAs font partie des plus préoccupantes, car ce sont des molécules biologiquement actives initialement conçues pour des applications de santé précise. La présence de ces substances dans l'environnement expose les organismes à des risques considérés comme toxiques [2]. À l'échelle mondiale, plusieurs classes de PPhAs (ex. : antibiotiques, analgésiques, anti-inflammatoires, etc.) ont été détectées et quantifiées dans différents compartiments aquatiques. Les concentrations reportées restent souvent de l'ordre du µg/L au ng/L [1]. Le tableau 2-1 présente un ordre de grandeur des concentrations de quelques PPhAs couramment quantifiés dans les milieux aquatiques. La présence de ces contaminants dans l'eau potable et d'irrigation pose également risque sanitaire et un défi de gestion de l'eau dans de nombreuses régions, notamment, dans les zones souffrant de pénurie d'eau potable et de mauvaise gestion des eaux usées [2].

Tableau 2-1. Ordre de grandeur des concentrations en ng L^{-1} de PPhAs dans les milieux aquatiques.

	Effluent STEP	Eau de surface	Eau souterraine	Références
Acétaminophène	11,1 - 59	25 - 127	2,1 - 1890	[54]; [55]; [56]; [57]; [58]; [59]
Bézafibrate	22,5 - 400		10	[60]; [61]
Carbamazépine	420 - 1510	36 - 114	72	[62]; [63]; [56]; [57]; [64]
Diclofénac	60,5- 263,5	10 - 60	15 - 55	[60]; [65];
Ibuprofène	30	34	2,12 - 988	[63]; [66]; [56]; [67]; [64]
Sulfaméthoxazole	65 - 102,25	82	9 - 113	[60]; [57]

En effet, ces molécules biologiquement actives dans l'environnement même à faible concentration peuvent être potentiellement dangereuses (ex. : perturbation endocrinienne, cancérigène, troubles hormonaux) pour les organismes non ciblés qui y sont exposés de façon chronique [2,68,69]. Par exemple, certains anti-inflammatoires non stéroïdiens (AINS) sont reconnus comme des substances perturbatrices du système endocrinien. Ji et al., ont observé une réduction de la fécondité et une diminution de la quantité d'œufs ainsi qu'un retard de l'éclosion chez des poissons-zèbres dus à des concentrations en ibuprofène ≥ 1 µg/L [70]. La présence de ces PPhAs dans l'eau potable et les eaux usées recyclées à des fins d'irrigation soulèvent aussi une question de santé publique [2]. De plus, certains PPhAs sont bioaccumulables. Celles-ci présentent donc un risque pour les organismes dans la chaîne alimentaire. Le déclin des populations de vautour au Pakistan, est lié à la consommation de carcasse de bœufs qui avaient été traités au diclofénac un anti-inflammatoire connu pour s'accumuler dans les organismes

[71]. Une autre préoccupation concerne l'effet synergique encore méconnu des mélanges de contaminants dans l'environnement d'autant plus qu'à ce jour, aucune norme ne fixe des limites réglementaires de concentrations pour les PPhAs dans l'environnement. L'absence de méthode de traitement efficace et l'augmentation de la production industrielle pour soutenir la croissance démographique suscitent des défis technologiques, sociopolitiques et sanitaires.

Plusieurs méthodes de traitement physicochimiques et biologiques ont été proposées ces dernières années pour l'élimination des contaminants organiques persistants dans l'environnement [72]. Les procédés de traitement biologiques représentent des stratégies durables et économiques qui consistent à exploiter les capacités métaboliques des organismes vivants (ex. : bactéries, champignons) pour la dépollution des milieux contaminés (ex. : eaux, sols). La mycoremédiation basée sur l'usage des champignons responsables de la pourriture du bois figure parmi les alternatives attrayantes de traitements biologiques [73].

2.2 Mycoremédiation des contaminants dans l'eau

Avec plus de 100 000 espèces répertoriées, le règne des champignons constitue plus de la moitié de la biomasse microbienne dans le sol (>2 tonnes/Ha contre <1 tonne/Ha pour les bactéries) [11]. Les familles des ascomycètes et des basidiomycètes représentent la majorité des espèces identifiées dans l'environnement. Ces espèces lignivores sont, entre autres, responsables de la pourriture du bois [11]. Selon leur capacité à dégrader les composés du bois, les basidiomycètes sont classés en différentes catégories parmi lesquelles ceux responsables de la pourriture blanche du bois (WRFs) [10]. Au moyen de leur machinerie métabolique unique constituée d'une variété de biomolécules (ex. : enzymes lignolytiques, médiateurs rédox), les WRFs ont la particularité de décomposer tous les polymères du bois, même les plus complexes et récalcitrants comme la lignine. Cette caractéristique leur confère un fort potentiel biotechnologique pour la bioremédiation des contaminants organiques persistants [11,74].

2.2.1 Mécanismes d'élimination et efficacité des WRFs

À l'image de la diversité des biomolécules qu'elles sécrètent, les processus impliqués dans la transformation des contaminants par les WRFs demeurent complexes et ne sont pas encore complètement établis [14]. Une des difficultés reste la distinction entre les mécanismes intra et

extracellulaires, qui interviennent dans l'élimination des contaminants. Les mécanismes connus engagés dans la mycoremédiation comprennent la sécrétion d'enzymes extracellulaires et intracellulaires ainsi que les phénomènes de biosorption (Figure 2-2). De plus, les WRFs peuvent sécréter des métabolites naturels qui peuvent jouer le rôle de navette lors du transfert des électrons entre les enzymes et les contaminants. Ce mécanisme contribue à élargir la gamme de contaminants dégradés [75]. L'utilisation des WRFs pour la bioremédiation présente l'avantage de bénéficier de l'ensemble de ces processus biochimiques et physiques.

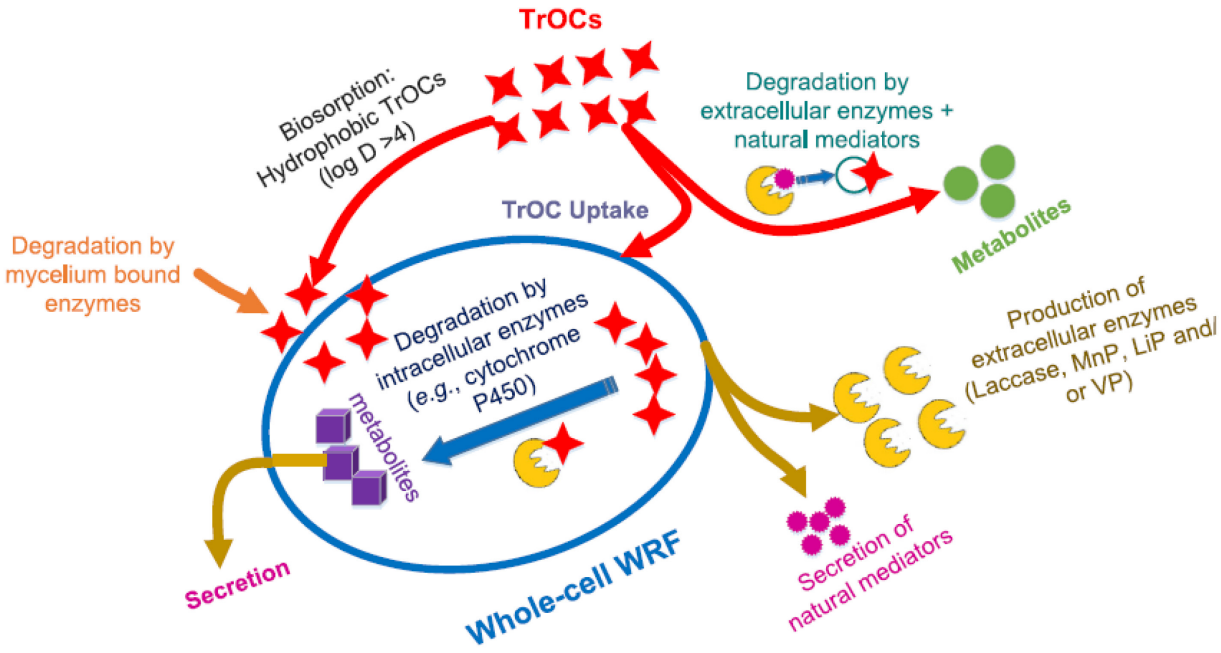

Figure 2-2. Schéma des principaux mécanismes fongiques impliqués dans l'élimination des contaminants (extrait de [76]).

Les WRFs ont été étudiés pour des applications variées de mycoremédiation. Les recherches ont démontré leurs capacités à éliminer des contaminants organiques persistants (ex. : HAPs, chlorophénols, colorants et PPhAs), dans des matrices complexes (ex. : les effluents municipaux, hospitaliers, des usines de pâtes à papier) [10,77–80]. Diverses études ont montré que divers champignons de la pourriture blanche, y compris *Phanerochaete sp*. et *Trametes sp.,* sont capables de minéraliser les hydrocarbures aromatiques polycycliques (HAPs), ce qui est en corrélation avec la production d'enzymes modifiant la lignine [81]. Par exemple, la forte demande chimique en oxygène (DCO) et la présence de composés phénoliques persistants dans

les eaux usées des moulins à huile d'olive constituent un problème environnemental majeur. L'étude de Ntougias et al. a permis d'évaluer l'aptitude de 49 souches à éliminer les phénols totaux dans ce type d'effluent préalablement stérilisé. Après 35 jours de traitement, l'ensemble des souches a permis d'éliminer au moins 60 % des phénols totaux et de réduire sa toxicité [19]. Marco-Urea et al. ont mis en évidence la capacité de *T. versicolor* à éliminer près de 91 % de l'acide clofibrique et 58 % de la carbamazépine dans un milieu réactionnel synthétique favorisant la croissance du champignon [82].

Une revue récente présente les procédés en continu de mycoremédiation à l'aide des deux souches de WRFs les plus étudiées (*P. chrysosporium et T. versicolor*). La plupart des traitements répertoriés utilisent des milieux de culture stériles, souvent synthétiques et avec ajout des contaminants issus de standards commerciaux à des concentrations généralement plus élevées que celles qui sont rencontrées dans les matrices environnementales [73]. Ces pratiques permettent d'évaluer le potentiel de mycoremédiation des contaminants dans des conditions optimales, car, elles empêchent la compétition vis-à-vis du substrat avec d'autres microorganismes, et offrent une meilleure reproductibilité des expériences [4].

Les chercheurs ont observé que l'efficacité du traitement de mycoremédiation dépend de paramètres tels que l'espèce de WRFs utilisée, les conditions d'opération (ex. : composition du milieu, pH, température) et les propriétés physicochimiques des contaminants [76,83]. De ce fait, la mise en place de procédé nécessite une sélection préalable des organismes. Le choix s'effectue généralement par des expériences de décoloration de colorants en milieu solide sur plaque d'Agar [84]. La sélection dans les matrices réelles devrait être privilégiée afin d'assurer la robustesse du traitement [20].

2.2.2 Limitations de la mycoremédiation

Malgré leur efficacité démontrée à l'échelle du laboratoire, les traitements de mycoremédiation des eaux utilisant les WRFs ne sont toujours pas appliqués à l'échelle industrielle. Les WRFs utilisent des mécanismes métaboliques secondaires qui permettent de dégrader les contaminants mêmes à faibles concentrations. Cependant, ces derniers ont besoin d'une source de carbone et de nutriments autres que les contaminants pour supporter leur croissance [11]. Les approches d'ajout de cosubstrat telles que le glucose et l'usage de conditions stérile pour les traitements de

mycoremédiation ne sont pas économiquement soutenables à grande échelle. Les substrats lignocellulosiques (ex. : bagasse, déchets de sciure) représentent une alternative bon marché pour la croissance des WRFs. Ces déchets contiennent du carbone, de l'azote et d'autres minéraux qui supportent la croissance des WRFs. De plus, ils permettent de simuler l'environnement naturel des WRFs [85]. L'exploitation de ces sources de carbone moins coûteuse est de plus en plus étudiée, et nécessite d'être optimisé. Ces stratégies devraient être à privilégier dans le but de développer des approches de traitement économique et durable.

La formation de biomasse pendant la croissance des champignons peut augmenter la viscosité du milieu et causer des problèmes de transfert de masse et d'oxygène dans le milieu. L'immobilisation du champignon sur des matériaux tels que le charbon actif, la mousse de polyuréthane, etc. constitue une technique qui peut être utilisée pour atténuer ces problèmes [86]. Une stratégie intéressante d'immobilisation consiste à recourir à des matériaux lignocellulosiques (ex. : copeaux de bois) [87]. Cette stratégie permet de tirer avantage du matériau comme support, et comme source de carbone à faible coût pour la croissance des WRFs. Récemment, la souche de *T. versicolor* a été immobilisée sur des copeaux de bois et appliquée en continu dans un réacteur à lit garni ruisselant. L'expérience a permis durant 49 jours d'éliminer un ensemble de PPhAs (ibuprofène, kétoprofène et naproxène) ajouté dans un effluent hospitalier non stérilisé [16]. Toutefois, pour des effluents industriels initialement riches en composés phénoliques, la dépolymérisation du substrat pourrait ralentir le traitement en augmentant le taux de phénol dans le milieu réactionnel [88].

Le temps de croissance assez long (plusieurs jours) reste un inconvénient majeur des traitements de mycoremédiation [17]. De ce fait, la mise en place d'un tel traitement dans une STEP apparaît inenvisageable pour le moment. Une revue récente suggère l'utilisation des WRFs pour des applications de prétraitement de niche, en particulier pour des effluents spécifiques avant leurs rejets dans le réseau d'assainissement ou dans l'environnement [73].

De plus, plusieurs auteurs, dont Ding et al. 2013, ont démontré la capacité de biosorption de contaminants tels que les HAPs par les WRFs. Les résultats de leur étude ont montré qu'après 3 jours d'incubation la biomasse vivante de *P. chrysosporium* a permis d'absorber plus de 50 % du pyrène en solution [89]. La principale contrainte de cette approche concerne également la gestion de la biomasse à la fin du traitement.

Certaines études se concentrent sur l'isolation et l'emploi des enzymes extracellulaires des WRFs. Cette approche permet d'éviter les temps de croissance longs des WRFs ainsi que des phénomènes de biosorption. L'utilisation des enzymes isolées offre également plus de contrôle vis-à-vis des conditions opératoires. Cependant, l'application des enzymes libres isolées dans des conditions environnementales dénaturantes (ex. : température, pH, et présence de toxines) peut entraîner une dénaturation ou une instabilité de ces dernières et ainsi réduire leur efficacité ou leur durée de vie [90].

2.2.3 Les enzymes fongiques d'intérêt en bioremédiation

Plusieurs enzymes extracellulaires sont impliqués dans les mécanismes catalytiques des WRFs. Les trois principaux enzymes d'intérêt en bioremédiation et quelques spécificités typiques sont présentés dans le tableau 2-2.

Tableau 2-2. Caractéristiques majeures des principaux enzymes extracellulaires d'intérêt [11,91,92]

Enzymes	Caractéristiques typiques et conditions optimales
Laccase (EC 1.10.3.2)	**Masse molaire** : 50-80 kDa / **Cofacteur** : O_2 / **Glycosylation** : 10-20 % **pH** : 3,5-6 / **Température** : 20-30ºC / **Potentiel rédox** : 0,3-0,8 V **Substrats** : phénoliques, non phénoliques
Lignine peroxydase (EC 1.11.1.14)	**Masse molaire** : 35-48 kDa / **Cofacteur** : H_2O_2 / **Glycosylation** : 20-30 % **pH** : ~3 / **Température** : 35-45ºC / **Potentiel rédox** : 1-1,5 V **Substrats** : phénoliques, non phénoliques
Manganèse peroxydase (EC 1.11.1.16)	**Masse molaire** : 32-62 kDa / **Cofacteur** : H_2O_2 / **Glycosylation** : 5-15 % **pH** : ~4 / **Température** : 30-40 ºC / **Potentiel rédox** : 0,8-1,5 V **Substrats** : non phénoliques

Ces trois oxydoréductases ont démontré une grande capacité pour la dégradation de contaminants phénoliques et non phénoliques dans l'eau (ex. : des PPhAs, perturbateurs endocriniens, des HAPs, chlorophénols) [25,91,93–95]. Par exemple, un extrait de LiP de *P. chrysosporium* a permis d'éliminer 100 % du diclofénac en solution, au bout de 2 h [96]. Tsutsumi et al. ont observé une élimination totale du bisphénol A et du nonylphenol en solution avec la MnP après 1 h de traitement [97]. Le MnP de *P. chrysosporium* a également permis

d'éliminer 99,4 % du triclosan dans l'eau après 1 h de traitement [98]. La laccase de *T. versicolor* a permis l'élimination d'un mélange de 10 PPhAs dans une solution. Le diclofénac, le naproxène et l'indométacine ont été complètement éliminés au bout de 2 h de traitement, suivi du kétoprofène (50 %) et de l'ibuprofène (37 %) au bout de 3 h de traitement [99]. Ces biocatalyseurs sont en cours d'évaluation pour la bioremédiation des contaminants dans l'eau [100].

Bien que leurs potentiels rédox soient plus élevés, l'utilisation à l'échelle industrielle des peroxydases est limitée par l'ajout de leur cofacteur (H_2O_2). En revanche, les laccases apparaissent plus avantageuses pour des applications à grande échelle, car, elles n'utilisent que l'oxygène moléculaire comme cofacteur.

2.3 Les laccases fongiques

Les laccases, sont des glycoprotéines multi cuivre oxydase, dont la production a été observée chez les plantes, champignons, bactéries et insectes [101]. À cause de leur potentiel rédox plus élevé, les laccases fongiques figurent parmi les plus utilisées dans les applications de bioremédiation.

2.3.1 Mécanisme catalytique et production

Ces dernières décennies, plusieurs auteurs ont proposé une description détaillée des structures et propriétés des laccases [102,103]. Brièvement, le mécanisme d'oxydation de la laccase s'effectue généralement par l'extraction d'un électron du substrat. Il en résulte la formation d'un radical libre avec la réduction de l'oxygène en molécule d'eau (Figure 2-3). Le cycle catalytique de la laccase repose sur les 4 atomes de cuivre qu'elle comporte. Ces atomes sont classés en trois types distribués entre différents sites. La catalyse d'oxydation du substrat s'opère en trois étapes. Le type de cuivre 1 (T1) est impliqué dans la capture des électrons issus de l'oxydation du substrat. Par la suite, le transfert des électrons vers la structure trinucléaire des cuivres de types 2 et 3 (T2 et T3) conduit à la réduction de l'oxygène en eau. La connexion entre les deux sites (T1 et T2-T3) est assurée par une connexion tripeptide d'acides aminés (His-Cys-His) (Figure 2-3).

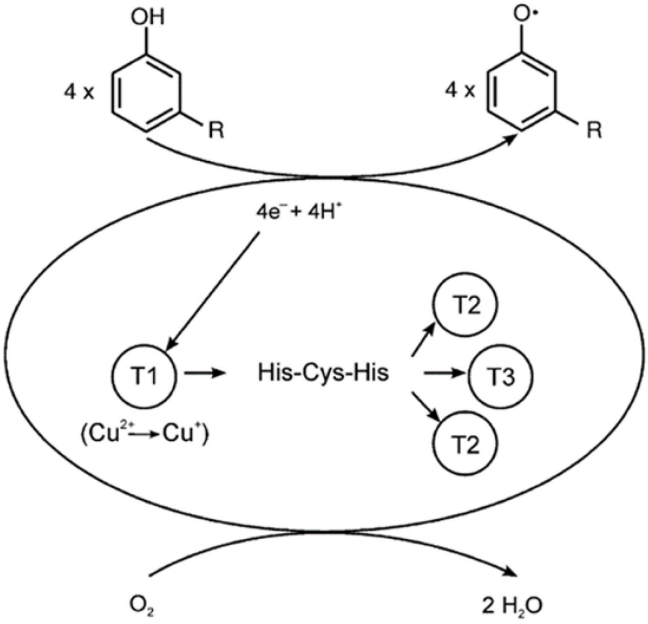

Figure 2-3. Représentation schématique du cycle catalytique d'oxydation d'un substrat phénolique par la laccase (extraite de [104]).

Les laccases sont essentiellement extracellulaires et sont sécrétées majoritairement par les basidiomycètes (ex. : *T. versicolor, P. chrysosporium, P. ostreatus*) [103]. Bien que la plupart des laccases purifiées soient des enzymes extracellulaires, les WRFs sécrètent également des laccases intracellulaires. Il a été suggéré que la localisation de la laccase est probablement liée à sa fonction physiologique [104]. Les laccases fongiques sont impliquées dans des processus tels que la sporulation, la production de pigments, la défense contre le stress, dégradation de la lignine… etc. La quantité de laccase sécrétée dépend du type de champignons, des conditions de culture ainsi que de la composition du milieu de croissance. Les basidiomycètes sont reconnus pour être les plus grands producteurs de laccase. Les groupes de *Pleurotus* et de *Trametes* font partie des espèces les plus étudiées, parmi lesquelles *P. ostreatus, T. versicolor,* ou *T. hirsuta* [103]. Le pH et la température optimaux pour la production de la laccase pour la majorité de ces souches se situent entre 4 et 6 et entre 25 °C et 30 °C, respectivement [74]. Le type de culture (submergée ou solide), la source de carbone, ou la présence d'agents inducteurs peuvent influencer la croissance des WRFs et la production de la laccase [105,106]. La méthode de fermentation submergée demeure la plus couramment employée [107]. Ce mode de croissance consiste à inoculer le champignon dans un milieu liquide avec les nutriments

nécessaires à sa croissance. L'usage d'un milieu liquide favorise la distribution homogène et une meilleure absorption des nutriments. L'un des principaux inconvénients de cette technique est la formation de mycélium pendant la croissance des champignons. Cela peut augmenter la viscosité du milieu et rendre ainsi difficile l'opération d'un agitateur lors de l'utilisation d'un bioréacteur agité pour la production. Le dysfonctionnement des agitateurs peut causer des problèmes de transfert de masse et d'oxygène dans le réacteur.

Ces dernières années, les techniques de fermentation solide ont beaucoup été étudiées pour la production de la laccase. La fermentation solide se fait en utilisant des matériaux synthétiques ou organiques comme substrats en l'absence ou presque d'eau libre [108,109]. Les substrats utilisés sont généralement des déchets lignocellulosiques riches en carbone, de l'azote et autre élément nécessaire à la croissance des microorganismes [110,111]. Les techniques de fermentation valorisant les déchets lignocellulosiques riches en carbone et autres éléments nécessaires à la croissance des WRFs sont de plus en plus étudiées pour la production économique de la laccase [85,112]. De nombreux groupes de recherche ont tenté d'améliorer la production de laccase à travers la sélection de souches en fonction du milieu de culture [113,114]. L'utilisation de déchets comme substrat est économiquement avantageuse et la technique est simple à mettre en place et consomme moins d'énergie. De plus, contrairement à la fermentation en milieu liquide, la fermentation solide reflète l'environnement naturel des WRF [115,116]. L'inconvénient principal de cette technique réside dans la difficulté de concevoir des bioréacteurs adaptés. La non-uniformité du milieu entraîne également une grande variation interbatch au niveau de la production d'enzyme [111,117].

La purification des enzymes emploie des méthodes coûteuses telles que la filtration sur gel, la chromatographie d'affinité. L'utilisation des enzymes purifiées pour les applications de bioremédiation à faible valeur ajoutée comme le traitement des eaux usées à grande échelle ne peut pas être économiquement viable. L'usage d'extrait brut d'enzyme est donc à prioriser pour ce type d'études. À ce jour, plusieurs applications utilisent encore des enzymes commerciales. En plus de réduire les coûts du procédé, les extraits bruts d'enzymes peuvent contenir d'autres biomolécules telles que les médiateurs naturels sécrétés par le champignon qui contribuent à élargir le spectre d'action de l'enzyme [76,83].

2.3.2 Versatilité de la laccase

La laccase a un potentiel redox inférieur à ceux des peroxydases. Cela restreint leur application pour l'oxydation directe de composés non phénoliques avec un potentiel redox supérieur au leur [75]. L'utilisation d'agents médiateurs redox permet de surmonter cette limitation. Les médiateurs sont des composés chimiques de faible poids moléculaire qui participe à élargir le spectre d'action de l'enzyme. Le médiateur est d'abord oxydé par l'enzyme avant d'agir comme transporteur d'électron entre l'enzyme et le substrat permettant ainsi l'oxydation du substrat (Figure 2-4). À travers le médiateur, l'enzyme peut oxyder des molécules présentant des structures complexes (ex. : lignine) [118].

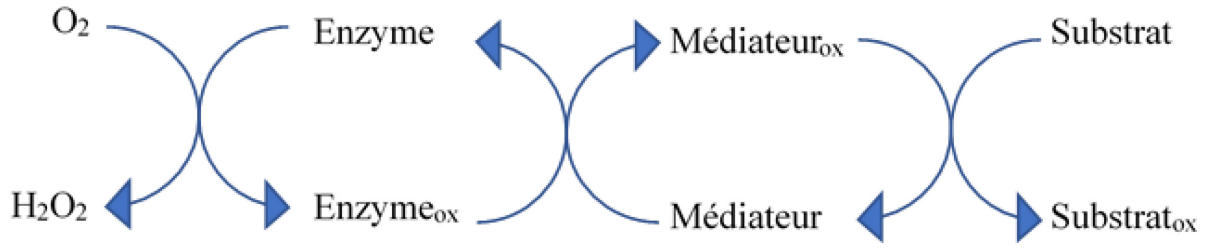

Figure 2-4. Représentation schématique du système enzyme-médiateur.

Les médiateurs peuvent être naturels (provenir de l'environnement (ex. : alcool vératrylique)). Ils peuvent également venir de substances synthétiques. Parmi les médiateurs les plus connus de la laccase, on compte le 2,2'-azino-bis-(3-éthylbenzothiazoline-6-sulfonic acid) (ABTS) et l'hydroxybenzotriazole (HOBt) [119]. L'efficacité du système enzyme-médiateur pour l'élimination d'un composé reste dépendante du type de médiateur et de la source de l'enzyme. Parra Guardado et al. ont récemment évalué le potentiel de la laccase provenant de 3 souches différentes pour l'élimination de PPhAs dans l'eau. Après 72 h de traitement, les trois laccases seules n'ont pas permis d'éliminer les molécules étudiées (amoxicilline, sulfaméthoxazole, ciprofloxacine et carbamazépine). Cependant, l'ajout de l'ABTS et du syringaldehyde a fourni des taux d'élimination allant de 50-100 % en moins de 3 h pour toutes les molécules sauf la carbamazépine [120]. La carbamazépine est un composé persistant et omniprésent dans l'environnement. Cette molécule est difficilement dégradable par la laccase [51,120]. Naghdi et al. ont démontré que les conditions de réactions peuvent influencer le taux d'élimination de ce type de composé. En présence d'ABTS, la laccase de *T. versicolor* a permis de dégrader jusqu'à 95 % de la carbamazépine à 35 °C et à pH 6 [121]. Cependant, ce type de traitement nécessite

d'être optimisé. De plus, la question reste posée quant à la faisabilité et la viabilité économique des médiateurs synthétiques assez coûteux comme l'ABTS.

Les radicaux produits durant la catalyse enzymatique de composés phénoliques permettent également à travers des réactions non enzymatiques (ex. : polymérisation) d'accroître le spectre d'action de l'enzyme [122,123]. Ces mécanismes ont permis d'expliquer l'élimination de molécules non phénoliques par la laccase [124–126]. Dernièrement, ces phénomènes ont conduit à une meilleure élimination de la carbamazépine en solution en présence d'acétaminophène par la laccase de *T. hirsuta*. En présence d'acétaminophène, cette laccase a permis d'éliminer 40 % de la carbamazépine contre 5 % en absence d'acétaminophène [127].

Malgré leurs efficacités démontrées au laboratoire, l'application à grande échelle des traitements enzymatiques est restreinte par les coûts élevés de production du biocatalyseur. La difficulté de récupérer et de recycler les enzymes constitue aussi un frein à leur utilisation sous forme soluble. De plus, la sensibilité des enzymes libres aux conditions opératoires souvent dénaturantes telles que celles rencontrées dans les eaux usées (ex. : variations de pH et de température, etc.) limite fortement leur utilisation.

2.4 Immobilisation des enzymes

Une enzyme immobilisée est une enzyme qui est maintenue par des moyens physiques et/ou chimiques dans un espace sans complètement altérer son activité catalytique. Selon les mécanismes impliqués, deux grandes techniques d'immobilisation existent : les méthodes physiques (adsorption, encapsulation) et chimiques (fixation par liaisons covalentes, réticulation) (Figure 2-5).

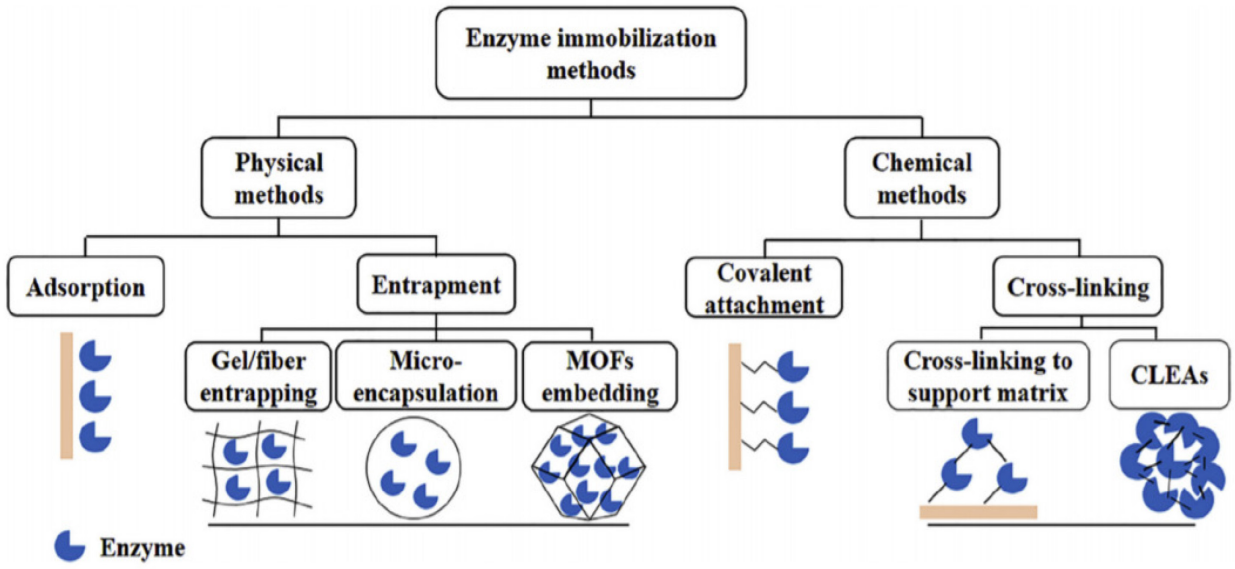

Figure 2-5. Représentation schématique des deux principales méthodes physiques (adsorption et encapsulation) et chimiques (fixation sur support par liaison covalente et réticulation) d'immobilisation et d'insolubilisation des enzymes. (illustration extraite de [128]).

Ces techniques permettent d'améliorer la stabilité des enzymes, ainsi que leur utilisation pour des applications en continu et de façon répétée [4,129]. Le choix d'une méthode dépend, des exigences attendues des propriétés du biocatalyseur (activité, stabilité), de la procédure d'immobilisation (degré de difficulté), de l'aspect économique (coût de production de l'enzyme, possibilité de recycler le biocatalyseur pour plusieurs cycles), etc. [130].

L'immobilisation des enzymes est un sujet qui a été amplement couvert dans la littérature. Succinctement, l'utilisation de solide pour l'immobilisation des enzymes conduit à la production de biocatalyseurs avec de grandes masses non catalytiques. Le support peut représenter plus de 90 % de la masse du biocatalyseur final, ce qui entraîne un faible rendement et une efficacité réduite du biocatalyseur [28]. Les approches de fixation irréversible peuvent conduire à la perte du support une fois l'enzyme désactivée, ce qui peut se révéler très coûteux à long terme pour des applications à grande échelle [131]. De plus, la fixation par liaisons chimiques des enzymes sur support peut produire des changements structurels au niveau de l'enzyme, conduisant en une modification (souvent une diminution) des propriétés catalytiques de cette dernière [132]. L'adsorption et l'encapsulation sont des méthodes qui n'altèrent pas la structure de l'enzyme. Les limitations de ces deux méthodes sont liées respectivement, à la faiblesse des interactions

entre l'enzyme et le support (ex. : interactions ioniques, forces de Van Der Waals) et aux limitations de diffusion du substrat (Figure 2.5). Il existe également un risque de relargage de l'enzyme adsorbée dû aux variations des paramètres physico-chimiques, ou à une perte de l'enzyme à travers la capsule poreuse [132].

Dernièrement, différents nanomatériaux organiques et inorganiques (ex. : nanotubes de carbone, nanoparticules) ont été proposés pour l'immobilisation des enzymes. Par exemple, certains auteurs ont mis en place des techniques utilisant des réseaux (organique et inorganique) de polymères poreux autour d'une enzyme pour former des *single enzyme nanoparticules* (SENs) [133,134]. Ces techniques nanométriques offrent une grande surface spécifique donc une charge protéique plus élevée et contribue à diminuer les transferts de masse. Ces méthodes ont participé à l'expansion du champ d'application des enzymes immobilisées dans des domaines comme la biomédecine, ou dans des applications industrielles chimiques particulières telle que l'hydrolyse de l'amidon [135,136]. En ce qui concerne les applications de bioremédiation des eaux usées à grande échelle, la difficulté de recyclage de ces nanomatériaux semble le principal frein à leur utilisation. Certains auteurs présentent les propriétés magnétiques comme une alternative à cette limitation, mais la faisabilité à grande échelle reste à démontrer [37].

Au regard des limitations des méthodes physiques, les techniques chimiques d'immobilisation par réticulation (ou insolubilisation) apparaissent intéressantes (Figure 2.5). L'insolubilisation s'effectue par le biais d'une réaction entre un agent de réticulation et les groupes fonctionnels des acides aminés des enzymes entre elles [130]. Cette technique permet de produire un biocatalyseur avec une plus grande proportion d'enzyme et évite des coûts supplémentaires liés au support [137]. Le mode de préparation des enzymes avant la réticulation diffère selon les techniques. Les méthodes de réticulation reposent sur la liaison des enzymes sous forme dissoutes entre elles, après cristallisation, après précipitation ou après séchage. Ces procédés aboutissent respectivement à la formation de *cross-linked enzymes* (CLEs), *cross-linked crystallized enzymes* (CLECs), de *Cross-linked enzymes aggregates* (CLEAs) et *Cross-linked spray dried enzymes* (CLSD). Ces techniques ont été décrites par plusieurs auteurs [28,29,137]. Parmi ces méthodes, les CLEAs offrent la meilleure alternative pour des applications enzymatiques comme la bioremédiation des eaux usées. En effet, cette méthode est simple à mettre en place et à optimiser [30]. Les CLEAs possèdent également une activité enzymatique élevée après insolubilisation (~70 % de l'activité initiale). De plus, cette méthode est compatible

avec l'utilisation d'extrait brut d'enzymes [138]. Plusieurs classes d'enzymes telles que les oxydoréductases (ex. : laccases, glucose oxydases), les hydrolases (ex. : lipases, protéases) ont été insolubilisées sous forme de CLEAs pour différentes applications, dont la l'élimination des TrOCs dans l'eau [129,139].

2.5 Les agrégats de laccases réticulées

La préparation des CLEAs se réalise en deux étapes simples qui sont : la précipitation des enzymes suivie de leur réticulation [138]. Plusieurs agents de précipitation des protéines tels que des sels, des solvants organiques miscibles à l'eau, etc. peuvent être utilisés [140]. L'un des agents précipitant le plus utilisé est le sulfate d'ammonium (SA), car il offre l'avantage d'être peu dénaturant pour les enzymes. Cette pratique souvent généralisée n'est pas sans conséquence sur le taux de précipitation de la protéine. L'étude de Matijošyte et al. 2010, souligne l'intérêt du choix de l'agent de précipitation, car celui-ci n'éprouvera pas la même efficacité selon la source de l'enzyme [30]. De même, plusieurs agents de réticulation (ex. : glutaraldéhyde (GLU), dextran polyaldehyde) ont également été décrits dans la littérature et peuvent être utilisés pour la préparation des CLEAs [141].

Dernièrement, l'évolution des techniques de préparation des lac-CLEAs à conduit à l'utilisation du chitosane (CHI), un biopolymère peu onéreux, disponible en quantité et respectueux de l'environnement [32,35]. La réticulation de la protéine avec le CHI s'opère par le biais d'un agent d'activation. Le l'éthyl-3-(3-diméthylamino-isopropyl) carbodiimide (EDAC) est l'agent carbodiimide le plus utilisé à cet effet [27]. Le mécanisme de réticulation en deux étapes consiste en la formation de liaisons covalentes entre les groupes fonctionnels carboxyles (-COOH) de l'enzyme et les groupes amines ($-NH_2$) du CHI, par l'intermédiaire de l'EDAC qui sert d'activateur (Figure 2-6).

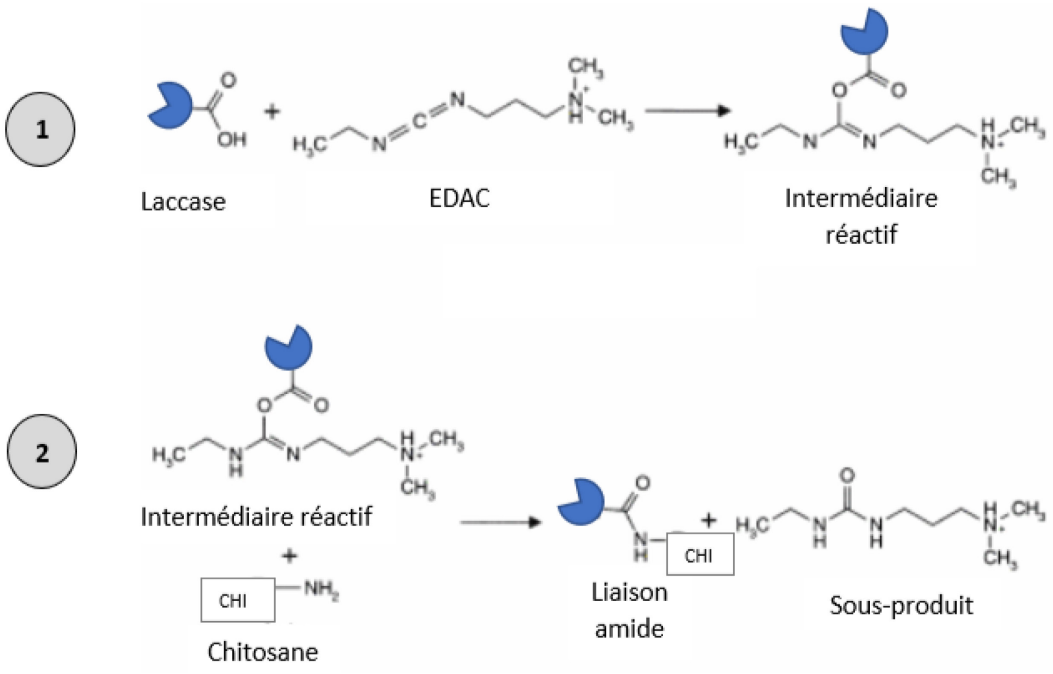

Figure 2-6. Étapes de réticulation de la laccase par le chitosane et l'EDAC (adapté de [142]).

Cette méthode a été appliquée avec succès ces dix dernières années pour l'insolubilisation de la laccase [33,35]. Il est difficile de comparer les études, car les résultats d'insolubilisation sont enzymes dépendants, et les conditions utilisées sont variables d'une expérience à l'autre. Néanmoins, de manière générale, la méthode CLEA a permis de renforcer la stabilité des laccases commerciales et des extraits bruts de laccase vis-à-vis des variations de pH, de température, mais aussi en présence de sels dénaturants et de solvants. Par exemple, la laccase de *S. putrefaciens* a pu être insolubilisée sous forme de CLEAs après précipitation avec du SA et réticulation avec le GLU. Le CLEAs formé a su maintenir 100 % de son activité initiale au-delà de 70 °C, tandis que l'activité de la laccase libre a chuté à partir de 55 °C. Les résultats ont également montré une amélioration des propriétés catalytiques des CLEAs (2 fois plus) que celles des laccases libres. Les auteurs ont par la suite évalué la capacité des CLEAs à décolorer une solution contenant un colorant utilisé dans l'industrie textile (le vert de malachite). Après 24 h de traitement en batch, le taux d'élimination était sensiblement le même (~90 % d'élimination) pour les laccases libres et les CLEAs [143].

La préparation des CLEAs entraîne la formation de particules insolubles amorphes de tailles variables (généralement entre 0,1 – 200 µm) [140]. La taille des particules des biocatalyseurs hétérogènes est un paramètre déterminant, en particulier pour l'efficacité du biocatalyseur, le

choix du réacteur adapté pour l'application continue et le choix de la méthode de recyclage. La vitesse de diffusion est influencée par la taille des particules, et celle-ci est déterminée, entre autres, par le rapport réticulant / enzyme. Des taux optimaux sont observés avec des particules plus petites, mais des considérations pratiques, par exemple la facilité de filtration, dictent l'utilisation de particules plus grosses [138]. La centrifugation reste le moyen le plus utilisé pour la préparation des CLEAs (collecte et lavage). Cette méthode est connue pour faire agréger les particules de CLEAs entre elles du fait du manque de résistance mécanique [138]. Cette agrégation entraîne une augmentation de la taille des CLEAs et pourrait accentuer les limitations de transfert de masse. Certains auteurs proposent des techniques de filtration membranaire pour le recyclage des CLEAs. Récemment, Ba et al. 2014, ont insolubilisé deux enzymes commerciales (la laccase et la tyrosinase) sous forme de combi-CLEAs. Les enzymes ont été précipitées en utilisant, le SA et réticulées avec du CHI après activation avec l'EDAC. En plus d'assurer la stabilité des enzymes, les combi-CLEAs ont été appliqués avec succès dans un traitement en continu à l'aide d'un bioréacteur membranaire durant 5 jours. Ce traitement a permis d'éliminer complètement un mélange de 14 PPhAs ajouté dans une eau usée municipale, avec une diminution de l'activité enzymatique des combi-CLEAs d'environ 30 % à la fin du traitement [33].

Malgré leur potentiel biotechnologique et les applications de traitement encourageant qui leur sont attribués, les CLEAs ne sont toujours pas appliquées à l'échelle industrielle, en particulier dans les bioprocédés environnementaux. La faible stabilité mécanique et les difficultés de recyclage des particules de tailles variables des CLEAs demeurent les principales causes [138]. Le recyclage des biocatalyseurs est facilité par l'augmentation de leur taille. Cependant, l'effet de la taille entraîne une diminution des propriétés cinétiques à cause des limitations de transfert de masse [140]. Par conséquent, pour atteindre une capacité opérationnelle et une robustesse satisfaisante face aux conditions opératoires, il est nécessaire de proposer de nouvelles stratégies d'amélioration de la stabilité et de recyclabilité des CLEAs.

2.6 Stratégies d'amélioration de la stabilité et de la recyclabilité des CLEAs

Plusieurs chercheurs ont travaillé l'amélioration de la stabilité et de la recyclabilité des CLEAs. Dernièrement, des stratégies, telles que la fixation d'un réseau polymérique poreux (ou *enzyme*

polymer engineered structure (EPES)) autour des CLEAs pour former des EPES-CLEAs [38], la fixation des CLEAs sur des nanoparticules magnétiques (M-CLEAs) [37] et l'encapsulation des CLEAs par piégeage dans des matrices polymériques poreuses (E-CLEAs) [39], ont été étudiées pour la stabilisation des lac-CLEAs.

Les considérations pratiques pour l'application des CLEAs à grande échelle pour le traitement des eaux usées nécessitent l'utilisation de particules plus grosses pour faciliter leur récupération à l'aide de techniques simples. Au regard des avantages et inconvénients de ces méthodes de stabilisation physiques et chimiques (Section 2.4), la méthode EPES apparaît comme la meilleure alternative pour l'amélioration de la stabilisation des CLEAs pour des applications environnementales de bioremédiation. Cette stratégie a été mise au point pour la première fois par Kim et Grate pour la stabilisation d'une enzyme (la chymotrypsine) [133]. La méthode consiste à fixer de façon covalente des structures de polymères avec les résidus d'acides aminés présents à la surface de l'enzyme. Ainsi une structure de polymères hybrides faits de vinyle et de silane (3-aminopropyltriethoxysilane (APTES)) a été fixée à la surface de l'enzyme étudiée, par polymérisation orthogonale sous un rayonnement UV. Cette préparation laborieuse a conduit à la formation de SENs de tailles nanométriques et a permis de stabiliser l'enzyme en augmentant de près de 280 fois sa demi-vie qui était initialement de 12 h [133]. Dans la foulée, Yadav et al. 2011, se sont inspirés de cette méthode pour stabiliser la carbonique anhydrase en simplifiant la méthode. Ces derniers ont procédé à la conjugaison de l'enzyme étudié avec le CHI et l'EDAC, avant de stabiliser le SENs formé par fixation de l'APTES. Exposés à différentes températures (-20, 4 et 20 ^{o}C), les SENs obtenus sont demeurés stables plus d'une centaine de jours [134]. Dans une étude plus récente [38], cette méthode a été adaptée pour la stabilisation des lac-CLEAs.

Dernièrement, les travaux de Hassani et al. 2014, ont conduit à la formation d'une nouvelle génération de CLEAs (ou EPES-CLEAs) [38]. Les EPES-CLEAs formés avec la laccase commerciale de *T. versicolor* ont été caractérisés dans des conditions contrôlées au laboratoire. Les résultats ont révélé des propriétés intéressantes pour la stabilisation des lac-CLEAs. Les EPES-CLEAs ont démontré une stabilité accrue entre 5 et 30 fois plus que les CLEAs face aux variations de pH, de température et face à la présence d'agents dénaturants [38]. Cette stabilité est liée à la rigidification du biocatalyseur par la fixation multipoints de réseaux de polymères

autour des CLEAs. Les résultats ont également montré une amélioration des propriétés catalytiques des EPES-CLEAs soit une augmentation 4 fois plus par rapport au CLEAs en utilisant l'ABTS comme substrat [38].

Toutefois, afin de confirmer le potentiel biotechnologique des EPES-CLEAs pour la bioremédiation des eaux usées municipales, la méthode EPES demande à être validée pour la stabilisation des CLEAs préparés à partir d'extrait brut de laccase. Cela est d'autant plus important, car les résultats obtenus avec un biocatalyseur sont difficilement attribuables à un autre. Des études complémentaires, portant sur la caractérisation de l'EPES-CLEAs dans des conditions environnementales appropriées de pH et de température et dans des matrices complexes réelles, permettront d'évaluer la robustesse de cette technologie.

2.7 Synthèse

La présence de contaminants organiques persistants dans l'environnement, en particulier, les PPhAs dans les eaux usées a conduit à la recherche de technologie de traitements versatiles. La stratégie de traitement à travers l'utilisation des WRFs et de leurs enzymes lignolytiques extracellulaires isolées a démontré une grande efficacité pour la dégradation d'une variété de molécules dans diverses conditions.

Les études réalisées au sujet de l'utilisation des WRFs pour des applications de bioremédiation des eaux usées ont permis de faire ressortir plusieurs restrictions pour des applications continues à l'échelle industrielle. La cinétique trop longue de ces procédés est identifiée dans la littérature comme le principal frein à l'exploitation des WRFs. De plus, le nombre de travaux effectués dans des conditions environnementales réelles demeure limité et difficile à réaliser. Ce champ de recherche est en plein développement. L'exploitation de cette approche prometteuse pour des applications de traitement d'effluent industriel particulier est suggérée. Toutefois, la complexité de ces matrices implique une sélection des WRFs les mieux adaptés au traitement.

Les travaux qui ont conduit à l'isolation et à l'utilisation des enzymes extracellulaires des WRFs pour la biocatalyse enzymatique des TrOCs dans l'eau apparaissent comme une alternative avantageuse pour contourner les limitations associées aux traitements fongiques. Cette approche offre un meilleur contrôle des conditions d'opération. Les choix de l'enzyme et de la stratégie d'immobilisation semblent toutefois importants et doivent être appropriés à l'application visée,

afin d'en assurer la faisabilité. La laccase et la méthode CLEA d'insolubilisation des enzymes ont été identifiées comme un biocatalyseur et une technologie versatile, économique, efficace et adaptée aux applications environnementales. Les recherches effectuées à ce sujet, suggèrent pour la plupart une nette amélioration de la stabilité des laccases avec même pour certaines, une augmentation des propriétés catalytiques de l'enzyme après insolubilisation. Toutefois, le nombre d'études réalisées dans des conditions représentatives de l'environnement reste limité. De plus, la robustesse des CLEAs doit être améliorée afin de résister aux conditions dénaturantes que peuvent représenter les matrices complexes et aussi assurer le recyclage du biocatalyseur par des méthodes conventionnelles simples. De même, l'évaluation des techniques de stabilisation dans des conditions environnementales s'impose pour une meilleure appréciation du potentiel réel des biocatalyseurs. Les stratégies de stabilisation des lac-CLEAs proposées restent insuffisantes et comportent plusieurs limites. Actuellement, la méthode EPES apparaît comme la meilleure stratégie de stabilisation. À ce jour, aucune étude n'a encore procédé à l'évaluation de cette méthode pour la stabilisation d'un extrait brut de laccase et son application pour la biocatalyse des TrOCs dans les eaux usées.

Les limitations et contraintes évoquées dans ce chapitre appellent, à développer les points suivants. À savoir : la mise en place de stratégies visant à renforcer la robustesse des approches de traitement par mycoremédiation et biocatalyse enzymatique des contaminants organiques persistants dans l'eau. Ce projet contribue au sujet à travers les points suivants : 1 – la présélection de souches de WRFs pour des applications futures de bioremédiation d'effluents industriels. 2 – l'adaptation de la méthode EPES pour des applications de bioremédiation environnementales à travers la valorisation d'un extrait brut de laccase insolubilisée sous forme de CLEAs.

Chapitre 3 - Application des lac-CLEAs pour la bioremédiation des PPhAs dans l'eau : Efficacité et défis à relever

3.1 Avant-propos

Ce chapitre a été publié dans le livre intitulé « *laccases in bioremediation and waste valorisation* ».

Titre français : Défis liés à l'application d'agrégats de laccase réticulés dans la biorestauration des polluants émergents des eaux usées.

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État : « publié; laccases in bioremediation and waste valorisation, SpringerNature microbiology monographs, Éditeur : Dietmar Schlosser ».

Contribution au document : Ce chapitre présente une revue sur l'application des lac-CLEAs pour l'élimination des PPhAs dans l'eau ainsi que des techniques utilisées pour améliorer les propriétés et le potentiel d'application des lac-CLEAs pour des applications de bioremédiation environnementales.

Résumé français : Les eaux usées municipales ont été reconnues comme la principale source de contaminants d'intérêt émergents tels que les produits pharmaceutiques et de soins personnels (PPSP) dans l'environnement. Les impacts de cette contamination sur l'environnement et la santé humaine ne sont pas encore entièrement connus. Les laccases sont des enzymes capables de dégrader divers PPSP. Cela a conduit à développer des biocatalyseurs prometteurs à base de cette enzyme. Les techniques d'immobilisation contribuent à améliorer la stabilité de l'enzyme en ce qui concerne la dénaturation et les conditions opérationnelles et permettent le recyclable du biocatalyseur. En raison de sa rétention d'activité élevée et de sa

simplicité, la technologie CLEA est considérée comme une méthode d'insolubilisation avantageuse, car elle n'utilise pas de supports physiques. La technologie CLEA a été appliquée avec succès pour la préparation de lac-CLEA. Cependant, des efforts considérables sont encore nécessaires pour améliorer la stabilité et la réutilisabilité des lac-CLEA, notamment pour leurs applications dans les procédés de traitement en continu. De nouvelles stratégies de stabilisation ont été développées pour surmonter ces limitations. Ce chapitre fait état des dernières stratégies mises en place pour la préparation et la stabilisation des lac-CLEA.

Title: Challenges in applying cross-linked laccase aggregates in bioremediation of emerging pollutants from wastewater.

Abstract: Municipal wastewaters have been recognized as the primary source of contaminants of emerging concern such as pharmaceuticals and personal care products (PPCPs) in the environment. The impacts of this contamination on the environment and human health are still not fully understood. Laccases are enzymes capable of degrading various PPCPs leading to widespread promising applications of laccases in bioremediation processes. Laccases are immobilized to broaden their applicability. Immobilization improves the enzyme stability regarding denaturing and operational conditions and creates a recyclable catalyst. Because of its high activity retention, stability, and simplicity, the cross-linked enzyme aggregate (CLEA) technology, has been considered as an effective carrier-free immobilization method. CLEA technology has been successfully applied for laccase insolubilization (lac-CLEA). However, considerable attempts are still needed to improve the stability and reusability of lac-CLEAs, especially in continuous treatment processes. Novel stabilization strategies have been developed to overcome the undesirable limitations of lac-CLEA applications in bioremediation. This chapter focuses on the latest improvements in the preparation and stabilization of lac-CLEAs for the removal of pollutants from waste streams.

3.2 Introduction

Pharmaceuticals and personal care products (PPCPs) are among emerging contaminants in water that have been commonly identified in the environment [1]. Municipal wastewater treatment plant effluents have been recognized as the primary source of these emerging pollutants in the environment [51]. Hence, existing WWTPs designed in the 19^{th} and 20^{th} centuries to treat

household wastewater and industrial effluents are not effective in removing these emerging contaminants [144].

The presence of PPCPs in wastewater is of interest in environmental pollution (aquatic and terrestrial ecosystems) because some PPCPs are biologically active even at low concentrations and have bioaccumulation potential [68,69,145]. PPCP contamination may lead to environmental and health problems. For example, male fish feminization as a result of exposure to steroidal hormones, development of antibiotic-resistant genes due to the release of antibiotic active compounds into the environment and various harmful effects on development or reproduction that may be caused by endocrine-disrupting chemicals (EDCs) [2,146,147]. The occurrence of these PPCPs in drinking water and irrigation water may also pose health concerns and wastewater management challenges in many regions, particularly in developing countries where wastewater treatment plants (WWTPs) are rare [148,149].

Furthermore, to face the problems of water scarcity in some regions of the world, the mobilization of alternative water sources, including wastewater recycling, is increasing [150]. Recycled wastewater is mainly used for non-potable direct and indirect uses, such as irrigation, watering green spaces, or industrial processes [148]. The impacts of these practices on the environment and human health are still not very well-known [151,152]. The health risks (chronic exposure to contaminants) and environmental risks (soil degradation, contamination of groundwater), present a gap to be filled to understand the impact of reusing wastewater containing emerging contaminants [153].

The progress in advanced wastewater treatment technology development has led to the creation of some innovative treatment processes. Bioremediation processes are considered one of the most effective and eco-friendly mechanisms for emerging pollutant treatment. Enzymatic technologies have gained interest because of their specificity and selectivity in removing contaminants from waste streams [100].

Enzymes are catalysts capable of carrying out specific reactions. Laccases (EC 1.10.3.2) and peroxidases (EC 1.11.1.7) are enzymes capable of degrading various PPCPs [154]. The effectiveness of enzymes has led to a focus on ligninolytic enzymes as a promising tool for biotransformation processes. Laccases have drawn attention in recent decades because of their high bioremediation potential [155]. Laccases are copper-containing oxidoreductase

glycoprotein widely distributed in fungi, bacteria, and plants. They have been broadly applied in numerous biotechnological and industrial fields [95,156]. Laccases are versatile catalysts capable of oxidizing a variety of phenolic and non-phenolic compounds and have been used in different operational conditions. They only need oxygen to oxidize their substrates, which is a significant economic advantage. The reaction end-products resulting in laccase degradation of phenolic compounds are also one of the advantages of laccase application in bioremediation processes. The resulting metabolites (reactive phenoxy radicals) form a variety of oligomers that have been found to significantly lose their biological activity and toxicity [27,157].

The economic viability of a large-scale application of enzymes in bioremediation is directly affected by the cost contribution of the enzyme to the treatment. So, to be cost-effective, enzymes must be simple to produce and handle, as well as stable and reusable, with high catalytic activity [121]. When suspended in an aqueous, the enzymes are soluble, making them challenging to reuse. The immobilization techniques consist of physical or chemical interactions to generate insoluble enzymes by confining them in a certain region of space. The immobilized enzymes are then reusable and contribute to reducing the treatment cost [158].

Physical immobilization methods consist of entrapment or binding enzymes onto a carrier material. The physical support reduces the volumetric activity of the enzymes compared with carrier-free immobilization approaches [135]. The most valuable carrier-free immobilization technique is the cross-linking enzyme aggregate (CLEA) method. The CLEAs are produced through covalent bonds that keep the enzyme aggregates together. CLEAs appear to have considerable bioremediation potential because of their high activity retention and stability, combined with their simple preparation protocol. CLEAs display a high catalytic property because the insolubilization method does not need physical support [28,138]. CLEA technology has been used for laccase insolubilization (lac-CLEA). Lac-CLEAs have been successfully applied to treat pollutants from different waste streams. However, to increase the probability of satisfying the operational requirements for large-scale applications, it is still necessary to improve the existing lac-CLEA preparation methods, to enhance the insolubilized laccase stability, as well as its catalytic properties. Better reactors adapted for the application of lac-CLEAS in real environmental and operational conditions need to be developed [33,100].

This chapter presents an overview of the latest strategies to enhance lac-CLEA formulation and stability enhancement to apply lac-CLEAs as a promising bioremediation tool for the removal of emerging contaminants from waste streams.

3.3 Cross-linked enzyme aggregate technology

Enzyme cross-linking technologies were developed in the middle of the 20th century. Three cross-linking methods using dissolved enzymes (CLEs), crystallized enzymes (CLECs) and aggregated enzymes (CLEAs) have been reported and extensively reviewed [28,137]. Among these methods, CLEA technology has shown many advantages for large-scale industrial applications. The method is simple and allows rapid optimization of the insolubilization conditions. CLEA technology provides a stable and recyclable catalyst with high enzymatic activity retention. Also, crude enzyme extract can be used to prepare CLEAs, which results in the formation of a low-cost biocatalyst [138]. CLEAs can be applied to a wide range of enzymes (including laccases) that can be combined to form combined cross-linked enzyme aggregates (Combi-CLEAs). A detailed description of CLEA technology has been exhaustively reviewed [137].

Briefly, CLEAs are obtained from precipitated enzymes forming aggregates that remain permanently insoluble after covalent binding with a cross-linking agent in a two-step process (Fig. 3-1).

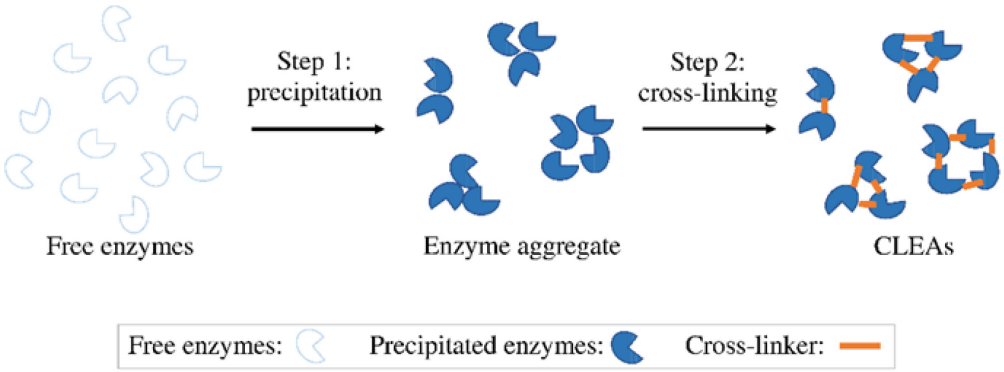

Fig. 3-1. Schematic representation of Cross-Linked Enzyme Aggregate preparation.

The first step in CLEA preparation consists of physical precipitation of the enzymes. The precipitation occurs by changing the hydration state of the molecules or by altering the

electrostatic constant of the solution [30]. The common precipitants involved in laccase aggregation are salts, organic solvents, or non-ionic polymers, such as ammonium sulphate, polyethylene glycol and tertbutyl alcohol) [30].

The second step is based on the formation of covalent bindings between the enzymes' primary amino groups of lysine and the reactive groups of the cross-linker [138]. Depending on the enzyme used, different cross-linkers can be used in CLEA formulation. Due to its availability and low cost, glutaraldehyde, a bifunctional aldehyde agent, is the most widely used cross-linker in CLEA preparation [138]. Glutaraldehyde reacts with the amino groups of the enzymes to form an amide bond that links the enzymes together. Other cross-linkers such as glyoxal, dextran polyaldehyde and the biopolymer chitosan, can also be used for lac-CLEA preparation [27].

The two-step procedure in CLEA preparation is simple. However, the combination of different enzyme properties, as well as the choice of precipitants and cross-linking agents can significantly turn this simple procedure into a more complex one that can result in numerous permutations. It is essential therefore, to determine the cross-linking condition from the start of the insolubilization process.

3.4 Determining the conditions for laccase aggregation and cross-linking

The two-step CLEA preparation procedure can affect the catalytic properties of the enzymes. These properties depend on the precipitating agent and the cross-linker used, as well as their concentrations [30]. Since, the precipitant and cross-linker can affect the conformation and rigidity of the enzyme which then changes the CLEA reactivity, they must be carefully chosen [30]. This choice depends on the biochemical properties of the enzymes. So, the best conditions must be defined experimentally and optimized to determine the concentrations needed for the enzymes' insolubilization. The optimum ratio of cross-linkers and enzymes is crucial to determine, especially when using an undefined crude enzyme preparation [30,122]. Generally, a low concentration of cross-linking agent is known to create insufficient bonds with the enzyme, while at a high concentration, it can increase the enzyme rigidity and prevent the substrate from reaching the active site resulting in lower apparent enzyme activity [122].

To reduce these adverse effects on the catalytic properties of lac-CLEAs, it is crucial to optimize the formulation conditions. Mathematical models using experimental designs are helpful in process optimization [159]. However, these empirical models do not allow for an in-depth characterization of the insolubilized enzymes. Other alternatives exist, such as the addition of polymers containing primary amino groups during the precipitation step to avoid excessive cross-linking of the enzyme. Bovine serum albumin (BSA) is mostly used as a source of protein and amino groups during lac-CLEA preparation. This technology can help increase the stability of the immobilized enzyme [122].

Optimization helps to reduce the use of the reagents and their undesirable effects on lac-CLEA properties and can also help to reduce the cost of the resulting insolubilized biocatalyst by reducing the reagent quantities to the minimum necessary, avoiding waste. During the optimization process, it is important to determine an equilibrium between the stability and apparent activity of the resulting lac-CLEAs, not to favor one parameter more than the others [32]. In some cases, laccase insolubilization might lead to a partial or complete loss of laccase activity because the reagents used during lac-CLEA preparation might interfere with the active site of the enzyme. Although glutaraldehyde remains an inexpensive and widely used cross-linker in laccase insolubilization, it is well known that it can contribute to enzyme inactivation during CLEA preparation. Glutaraldehyde can modify the active site residues of the enzyme. So, it is crucial to find solutions to prevent the cross-linker from entering the active site or inducing extreme conformational changes. Mild insolubilization conditions must be employed, or the reagents must be replaced, to prevent such problems.

Only a few studies report perspectives about the investigation of new cross-linking agents in lac-CLEA formulation for a sustainable biocatalyst preparation. A dextran-based cross-linker has been developed and successfully applied in CLEA preparation from several enzymes (penicillin G acylase, hydroxynitrile lyase, alcohol dehydrogenase, and two different nitrilases) [160]. The dextran molecules are too large to penetrate the active site of the enzyme, and CLEAs exhibited a higher enzyme activity. This technique has not been applied in lac-CLEA preparation yet.

Recently, chitosan was successfully tested as a cross-linking agent for laccase from *C. polyzona* insolubilization. The results showed hyperactivation of the lac-CLEA-based chitosan

conjugation formed. This overexpression can come from the conformational changes in laccase during the conjugation reaction by locking the enzyme into a more favorable conformation [32,35].

Most of the studies about lac-CLEAs have focused on characterization and batch experiment applications (Table 1). The shape and size of the CLEA particles are essential characteristics of a direct effect on the biocatalyst catalytic properties and recyclability. The structural organization of CLEAs is influenced by the cross-linking time. Generally, the particle size of CLEAs varies between 0.1 and 200 μm [140]. Although CLEA structure is known to significantly influence the effectiveness on solubilization of the insolubilized biocatalyst, this parameter is not often evaluated, and information on the lac-CLEA structure is still missing (Tables 1 and 2).

3.5 Lac-CLEAs in bioremediation: promises, efficiency, and drawbacks

In addition to being a low-cost process, one of the benefits of bioremediation is that it results in the generation of less toxic reaction end-products compared to chemical treatment processes [161]. A significant number of PPCPs are phenolic compounds, oxidable by laccases. The oxidation reaction involving laccases leads to the production of water and free reactive phenoxy radicals. These radicals are likely to polymerize and precipitate from the reaction solution [125]. The polymerization implies the inactivation of their biological activity and inhibits their action on living organisms [162].

Acetaminophen, a model phenolic pharmaceutical compound, has been frequently employed to study this reaction mechanism in order to propose reaction pathways involving laccases in the degradation of phenolic PPCPs [125,126]. Laccases have low redox potential, which prevents them from degrading non-phenolic compounds. However, some small molecules can act as an electron shuffle (laccase mediator system) and allow the transformation of non-phenolic compounds. Cross-coupling phenomena between the reactive phenoxy radicals and the non-phenolic compounds can also contribute to the elimination of non-phenolic compounds by laccases [127,163]. These reaction mechanisms expand the range of contaminants that can be oxidized by laccases, making this biocatalyst a promising tool for bioremediation processes.

Lac-CLEAs have been prepared from a wide variety of laccases in the past decades and efficiently applied to remove a variety of contaminants (Table 1). PPCPs have been successfully removed from aqueous solutions in batches and continuous experiments using lac-CLEAs. A crude enzyme extract from *C. polyzona* lac-CLEAs was prepared with glutaraldehyde and applied in a fluidized bed reactor to treat EDCs. The biocatalyst removed more than 95% of EDCs in 150 minutes [122]. Later, a new cross-linking method was proposed using chitosan to prepare lac-CLEAs [32,35,125]. In a six-hour batch experiment, Cabana et al. showed that the chitosan-based lac-CLEAs were able to remove 100% of triclosan from an aqueous solution, while the free laccases were less efficient with a 60% removal rate [35]. The same CLEA technology was applied in batches and continuous experiments to remove pharmaceutical compounds from wastewater. Acetaminophen and mefenamic acid were eliminated from the filtered wastewater in 24 h, and up to 93% of carbamazepine, a more recalcitrant compound, was eliminated after 72 h [164]. Lac-CLEAs have also been efficiently applied for PPCP elimination from wastewaters and other biotechnological applications such as dye decolorization from solutions (Table 3-1).

The efficiency of the laccase-based treatment process can be improved by enhancing its selectivity using the Combi-CLEA method [165]. Combi-CLEAs have the same economic and environmental benefits as CLEAs, with the difference that combi-CLEAs can target a greater variety of substrates, specific to each co-immobilized enzyme (Table 1). This combination system offers significant advantages in bioremediation. However, it is crucial to rigorously optimize the insolubilization conditions to respect the specificity of each enzyme. Laccases were successfully co-immobilized with two other oxidative enzymes, namely versatile peroxidase and glucose oxidase [139]. This combi-CLEA contributes to degrading a plethora of pharmaceutical compounds in laboratory conditions in solution and removes 25% of detected acetaminophen in real municipal wastewater in a batch experiment. Ba et al. 2014 tested the efficiency of a combi-CLEA of laccase and tyrosinase for the transformation of acetaminophen in wastewaters. In batch mode, the combi-CLEA transformed from 80 to 100% of acetaminophen from the municipal wastewater and more than 90% from a hospital wastewater [125]. Mass spectrometry detection of acetaminophen transformation products showed the formation of its oligomers as dimers, trimers, and tetramers due to the laccase and 3-hydroxyacetaminophen due to the tyrosinase.

Table 3-1. Lac-CLEA preparation and efficiency from contaminant bioremediation from waste streams.

Laccase sources	Precipitants / Cross-linkers	Lac-CLEA activity (Substrate)	Structures	Experiment conditions and pollutant removal rate.	References
Crude laccases from C. polyzona	Polyethylene glycol / Glutaraldehyde	148 U/g (ABTS)	BSA-free: 1 to 5 μm. With BSA: 100 to 200 μm	Continuous elimination: Fluidized bed reactor. Elimination after 50 min.: Nonylphenol = 90%; Bisphenol A = 30%; Triclosan = 90%. After 150 min.: > 95% elimination for all compounds.	[122]
Crude laccases from C. polyzona	Polyethylene glycol / Glutaraldehyde	148 U/g (ABTS)	-	Continuous elimination: perfusion basket reactor (BR). EDC elimination after 325 min.: 85%. Stable performances of the BR over 7 days.	[166]
Laccases from T. versicolor, T. villosa and A. bisporus.	Different precipitants / glutaraldehyde. Best precipitants: Polyethylene glycol 3400 (PEG 3400); Ammonium sulphate AS); 1,2 Dimethoxyethane (DME).	(Metol) T. versicolor: 17.6 U/mg (PEG 34000 at 20 ºC). T. villosa: 9.6 U/mg (AS at 4 ºC). A. bisporus: 3.6 U/mg (DME and AS at 4 ºC)	-	A batch experiment of T. versicolor lac-CLEAs with TEMPO as a mediator. After 5 h: Benzyl alcohol: 80%; 1-pentanol: 7%; 1-hexanol: 15%. After 20 h: 1-heptanol: 55%; 1-octanol: 34%; 1-nonanol: 13%; 1-decanol: 21%.	[30]
Laccases from T. versicolor	Tris buffer / Chitosan/ EDAC	626 U/g (ABTS)	-	6 h batch experiment. Triclosan removal: Free laccase = 60%; Lac-CLEAs =100%.	[35]
Crude laccases from C. polyzona	Ammonium sulphate / Chitosan/EDAC	737 U/g (ABTS)	1694.3 nm to 2313.7 nm	-	[32]
Laccases from S. putrefaciens	Ammonium sulphate / Glutaradehyde	(ABTS)	-	24 h batch experiment. Malachite green dye decolorization. Free laccase and Lac-CLEAs ≈90%.	[143]
Three oxidative enzymes: laccase (Lac) from T. versicolor, versatile peroxidase (VP) from B. adusta and glucose oxidase (GOD) from A. niger.	Ammonium sulphate / Chitosan_EDAC	30% of initial laccase activity was recovered along with 40% for each of VP and GOD. Lac (ABTS); VP (veratryl alcohol); GOD (qualitatively estimated through the increase of Mn-oxidizing activity of VP).	-	Batch experiments with free enzyme ability to degrade, a pharmaceutical compound (acetaminophen, naproxen, mefenamic acid, indometacin, diclofenac, ketoprofen, caffeine, diazepam, ciprofloxacin, trimethoprim, fenofibrate and bezafibrate, carbamazepine and its by-products 10–11 epoxy-carbamazepine) in synthetic wastewater. More than 80% elimination for the first five compounds. Combi-CLEA mediated removal reached up to 25% contained in real municipal wastewater effluent.	[139]
Laccases from T. versicolor (TvL) and Mushroom tyrosinase (Tyr).	Ammonium sulphate / Chitosan_EDAC	Combi-CLEAs-TvL: 12.3U/g (ABTS). Combi-CLEAs-Tyr: 167.4 U/g (L-DOPA).	-	Batch experiment: transformation of 80% to ≈100% of acetaminophen from the municipal wastewater and more than 90% from a hospital wastewater. Acetaminophen metabolites: oligomers as dimers, trimers and tetramers due to the laccase and 3-hydroxyacetaminophen due to the tyrosinase.	[125]

Laccases from T. versicolor (TvL)	Ammonium sulphate / Chitosan_EDAC	(ABTS)	-	Batch experiment + hybrid bioreactor. Acetaminophen and mefenamic acid ≈ 100% in 24 h; carbamazepine 93% in 72 h, from wastewater.	[164]
Laccases from Cerrena sp.	Ammonium sulphate / Glutaraldehyde	Activity recovery: 68.1% (ABTS)	-	With 80 mM NaCl, over 90% decolorization was obtained with free and immobilized laccases in 4 and 2 h, respectively.	[167]
Laccases from F. fomentarius and T. versicolor	Ammonium sulphate / Glutaraldehyde	-	-	Batch experiment: decolorization of Malachite green, Bromothymol and Methyl red dyes within 10 h. T. versicolor lac-CLEAs: 95% and F. fomentarius lac-CLEAs: 90%.	[141]
Laccases from T. versicolor (TvL) and Mushroom tyrosinase (Tyr)	Ammonium sulphate / Chitosan_EDAC	Combi-CLEAs-TvL: 12.3U/g (ABTS). Combi-CLEAs-Tyr: 167.4 U/g (L-DOPA).	-	Continuous elimination of 14 pharmaceutical compounds (PPs) from municipal wastewater using a hybrid bioreactor (HBR). Complete removal of all PPs after a 5-day continuous operation. The Combi-CLEAs retained ≈ 70% of its initial enzymatic activity after the treatment.	[33]

Lac-CLEAs are efficient in eliminating PPCPs from aqueous solutions and have many advantages for bioremediation applications (Table 3-1). However, the lack of a biocatalyst production cost estimate as well as the lack of understanding of the heterogeneous catalytic reaction mechanism and the need for the development of suitable reactors are some drawbacks for a large-scale application of lac-CLEAs.

Laccase insolubilization has a significant influence on the catalytic properties of the biocatalyst. Contrary to free laccases, lac-CLEAs are heterogeneous. The reaction occurs where the laccases are located (on the surface or within the insoluble structure of the CLEAs), while the substrates (contaminants) and the reaction products are dissolved in the reaction solution [168]. The catalytic parameters of free and insolubilized laccases are generally measured by determination of Michaelis-Menten kinetic constants with 2,2'-azino-bis(3-éthylbenzothiazoline-6-sulphonique (ABTS) as substrate (Table 1). Syringaldazine is another model substrate used for laccase activity assay [169]. The assays using these model substrates are simple, accurate and reproducible. However, for an efficient characterization of the biocatalyst, none of them reflects the laccase activity appropriately compared to a real substrate (e.g. the targeted PPCPs). The choice of a more effective method and substrate for laccase activity assay could help to better understand how the biocatalyst reacts to the target contaminants. In the case of oxidase catalysts, the activity can be assessed by monitoring the change in pressure in the reaction system [170]. Recently, this method was developed by [171], to measure laccase activity and provides an alternative to the commonly used spectrophotometric assays. However, the necessity to use high substrate concentrations with high molar extinction coefficient, and the use of a substrate with interfering biocatalytic products in the target UV-vis spectrum, are some of the limitations to the method [171]. Another promising alternative consists of assessing the enzyme kinetics using mass spectrometry combined with liquid chromatography (HPLC-MS). The latest method developed using this technique provided an accurate kinetic parameter measurement of laccases from *T. versicolor* and *G. lucidum*. Natural phenolic compounds (p-coumaric, ferulic and sinapic acid, and a lignin model OH-dilignol) were employed as substrates for laccase kinetic determination by HPLC-MS. The new method has been reported to be highly suitable and accurate for assaying laccase activity. This method also has the advantage of concomitantly examining laccase oxidation product profiles [172]. These examples are some interesting ways that must be explored more in order to better characterize lac-CLEAs.

In addition to the importance of choosing the best method for measuring laccase activity, it is also important to take into consideration that the kinetic properties of the insolubilized lac-CLEAs are influenced by their structure (shape and size), which can induce mass-transfer limitation phenomena. Most of the time, these diffusion phenomena are mingled with the enzyme inhibition caused by the insolubilization process, as discussed in the previous section. The lac-CLEA activity reported in the literature reflects the apparent activity. The intrinsic activity can be approximatively obtained by increasing the substrate diffusion through the heterogeneous structure, by promoting nano-sized aggregates. CLEA structure and size can be tuned during the insolubilization process to overcome this problem. The stirring speed and time during the biocatalyst washing steps can be optimized to avoid CLEA aggregation to form "clusters" (CLEAs size > 1 μm). Aggregates can also be formed during the CLEA recuperation by centrifugation or filtration. The aggregation increases the size of the CLEAs and then increases mass-transfer limitations [140].

One strategy consisting of the use of a magnetic field to facilitate CLEA recovery has been developed and proposed as an alternative to avoid the drawback of aggregation caused by centrifugation or filtration. [37] successfully immobilized laccase to form a stable and rigid structure of magnetic CLEAs (M-CLEAs). The catalytic efficiency of laccase was significantly improved, and the M-CLEAs were effectively applied in batch and continuous experiments of dye degradation in solution. The results showed more than 60% and 90% decolorization, respectively. More recently, Arca-Ramos et al. also demonstrated the catalytic potential of M-CLEAs to transform various phenolic and non-phenolic pharmaceutical active compounds present in solution. The developed biocatalyst showed good operational stability after ten successive batch reactions, maintaining up to 70% of the initial activity [124]. These results showed that M-CLEA technology provides a remarkable means to efficiently recover and maintain the catalytic properties of lac-CLEAs.

Nguyen et al. 2017 developed a novel strategy consisting of a multifluidic reactor carrying two coaxial laminar flows to create hollow cross-linked aggregates of laccases (h-CLEAs), by using acetonitrile and an aqueous solution. This method creates uniform h-CLEA sizes (220 nm) and provides high stability to the laccase. The h-CLEAs were able to degrade trypan blue dye molecules under an alkaline condition in a batch and continuous membrane treatment [173].

In addition to all these newly developed technologies to enhance lac-CLEA catalytic properties, a simple method consisting of co-precipitation of the enzyme with starch, followed by the cross-linking and removal of the starch by α-amylase to form porous CLEAs (p-CLEAs) was proposed. This method helps to decrease mass-transfer limitations [174]. A p-CLEA of laccase was successively prepared using a three-partitioning method. The resulting p-CLEA exhibited improved storage stability. The porous biocatalyst was successfully recycled 15 times [175].

All these preparation methods succeed in improving lac-CLEA non-catalytic (shape, size) and catalytic (activity, recyclability) properties for bioremediation (Table 3-2). Furthermore, to obtain a robust biocatalyst for waste stream bioremediation, lac-CLEAs must be highly stable.

3.6 Enhancement of lac-CLEA stability for bioremediation applications

CLEA technology improved the stability of laccases regarding denaturing conditions such as pH and temperature variation, as well as the presence of salts and organic solvents (Table 3-2). The effectiveness of lac-CLEAs for PPCP elimination from aqueous solutions has been widely proved. However, large-scale applications do not exist yet. One of the reasons is that efforts are still needed to improve lac-CLEA stability. A robust biocatalyst is necessary for the long continuous operation process in denaturing conditions like the conditions found in wastewater.

Besides the methods previously presented, new strategies focused on the stabilization of lac-CLEAs have been developed. CLEAs are compatible with some existing technologies such as encapsulation, silica coating, or polymer-engineered structure techniques that could provide resistance to operational stress factors [36].

Hence, the well-known and easy method of immobilization of enzymes by entrapment in a solid polymeric matrix is suitable for CLEA stabilization and easy recovery. A crude laccase extract from *T. pubescens* was cross-linked with glutaraldehyde before entrapment into Ca-alginate beads [39]. The resulting entrapped lac-CLEAs contributed to increasing the immobilization yield by 30% and reducing the catalyst leakage from the beads by 7- fold compared to entrapped free laccases. The entrapped lac-CLEAs were able to remove 99% of bisphenol A from an aqueous solution in batch experiments. The efficiency of the biocatalyst remained high (70%) after ten successive batch treatment cycles.

Another strategy, recently developed for lac-CLEA stability enhancement, consists of the formation of a polymeric network to stabilize the insolubilized biocatalyst. The resulting biocatalysts were named EPES-lac and EPES-CLEAs for Enzyme Polymer Engineered Structure using free laccase and lac-CLEAs [38], respectively. This strategy was developed based on the sustainable method of lac-CLEA formulation using the biocompatible cross-linking agent chitosan which was successively developed and optimized by [35] and [32]. The resulting EPESs were made from a commercial laccase from *T. versicolor*. The enzyme was surrounded by a polymeric network made of chitosan and 3-aminopropyltriethoxysilane (APTES). This stabilization method contributed to improving the thermoresistance of EPES-lac and EPES-CLEAs 30 times more than free laccases and lac-CLEAs. The stabilized biocatalyst also showed higher resistance than the free laccase and lac-CLEAs to denaturing conditions involving salts and organic solvents. However, the effectiveness of EPESs to treat contaminants in solutions has not been studied yet.

It is also interesting to consider the structure of the EPESs. The scanning microscopy of EPES-lac and EPES-CLEAs revealed a spherical shape for EPES-lac with an average size of 100 μm while the EPES-CLEAs showed an amorphous shape, which is consistent with the previous works about lac-CLEAs prepared with chitosan [32,35]. These results showed that the formulation conditions influence the structure of the EPESs.

The described approach for lac-CLEA stabilization, ensured a robust and easily recoverable catalyst, with excellent potential for the removal of emerging contaminants from waste streams. However, as is well-known, the final goal of immobilizing enzyme is to apply the recyclable biocatalyst for operations involving reactors. Further studies are needed to understand the behavior, particularly the mass-transfer limitations induced by the stabilization methods and resistance of lac-CLEAs, in a continuous reactor operation.

Table 3-2. Developed strategies for the improvement of catalytic properties as well as the stability of lac-CLEAs.

Enzymes	Immobilization method Precipitant/Cross-linker	Laccase activity (Substrate)	Particle size	Pollutants/Removal rate	Stability	References
Laccase from P. ostreatus	p-CLEAs. Three-phase partitioning co-precipitation technique of laccase and starch, followed by cross-linking with glutaraldehyde.	16 U/mg (ABTS)	Spherical shape with pores. p-CLEAs with 0.2% starch ≈ 0 – 81 nm.	Batch experiment elimination of triphenylmethane and reactive dyes by 60 – 70% respectively.	The half-life of free laccase at 55 ºC was calculated to be 1.3 h, while p-CLEAs did not lose any activity even after 14 h. p-CLEAs exhibited improved storage stability, catalytic efficiency and could be recycled 15 times with 60% loss of activity.	[175]
Laccase from T. versicolor	EPES-lac and EPES-CLEAs. Free laccase and lac-CLEA stabilization through a polymeric network of chitosan and 3-aminopropyltriethoxysilane (APTES). Ammonium sulphate /Chitosan_EDAC.	Apparent specific activity of laccase and CLEAs dropped from 1110.6 U/g to 34.3 U/g and 55.7 U/g to 11.5 U/g respectively (ABTS).	EPES-lac ≈ 100 nm (spherical shape). EPES-CLEAs was amorphous and relaxed rather than compact and uniform.	-	Optimal pH and temperature: Free laccase: pH 3 / 60 ºC EPES-lac: pH 4 / 20-60 ºC CLEAs and EPES-CLEAs: pH 4 / 60 ºC. Residual activities: After 22 days: EPES-lac and EPES-CLEAs ~300%. Free laccase and CLEAs lost 10% and 25% respectively after 24 h of incubation and were completely denatured after 7 and 9 days. In the presence of organic solvents: EPES-lac and EPES-CLEAs showed more than 100% of their initial activity, while free laccase and CLEAs lost 95% and about 30% of their initial activity respectively. In the presence of ZnCl2 and EDTA, free laccase, CLEAs and EPES-lac activities dropped to 5, 35 and 50%, respectively while EPES-CLEAs maintained nearly 100% of their activity.	[38]
Laccase from T. versicolor	M-CLEAs. Amino-functionalized magnetic nanoparticles bonded to lac-CLEAs. Chilled propanol / glutaraldehyde.	Activity recovery ~ 32% (ABTS).	M-CLEAs = 48 – 74 nm with few agglomerated particles.	A laboratory-scale perfusion basket reactor (BR) application for continuous decolorization of dyes.	Optimal temperature and pH: Free laccase: 40 ºC / pH 4 Lac-CLEAs: 4 ºC / pH 5 M-CLEAs: 30 ºC / pH 6	[37]

				61 to 96% of brilliant blue R, malachite green and reactive black 5 removal in 10 h.		
Laccase from T. versicolor	h-CLEAs. Preparation in a millifluidic reactor with two coaxial laminar flows. Acetonitrile/glutaraldehyde	0.26 U/mg (ABTS)	~220 nm	Trypan blue dye was decolorized within 270 min.	Free laccase optimum pH = 4. Free laccase retained around 42% and 13.9% of its optimal activity at pH 7.0 and 10.0, respectively. h-CLEAs retains about 90.4% and 80.9% of its activity at pH 7.0 and 10.0, respectively. h-CLEAs laccase can be trapped in a membrane for continuous degradation of trypan blue up to 96 h without losing any activity.	[173]
Crude laccase from T. pubescens	E-CLEAs (Entrapped CLEAs) E-lac (Entrapped laccase) Laccase was cross-linked with glutaraldehyde before entrapment into Ca-alginate beads.	Immobilization yield (> 72%) than that of the laccase immobilized only by entrapment (61.6%), (ABTS).	-	E-CLEAs: 99% removal of Bisphenol A (BPA) from an aqueous solution in 2 h in a batch experiment. BPA was removed in 10 successive batches with higher than 70% efficiency at the end of the last batch.	Thus, the free laccase: pH 3.0 and E-CLEAs: pH 4.0. Both catalysts showed optimal activity at 40 °C. At 70 °C, E-lac and E-CLEAs showed a relative activity of 49.1% and 54.6%, respectively, while the free laccase retained only 16.8% of its initial activity.	[39]
Laccase from T. versicolor	Entrapped lac-CLEAs: E-CLEAs. Entrapment carrier: porous silica Ammonium sulphate/Glutaraldehyde	E-CLEAs: 6.29 U/mg (ABTS). ≈50% specific activity loss upon the enzyme insolubilization.	Granulated appearance.	40 min batch experiment with complete removal of total phenol in solution.	Thermal and pH stability and activity retention in hydrophobic and hydrophilic solvents. Good operational stability and reusability: > 79% of initial activity after 20 cycles of successive operations.	[34]

3.7 Continuous reactors designed for lac-CLEA-based bioremediation

For economic and technical reasons, enzyme-based processes require reuse of the biocatalyst in long-time continuous operations. Different reactor configurations ranging from stirred tank to column-type reactors can be applied to enzyme reaction processes either in sequential batch operation or continuously (Fig. 3-2).

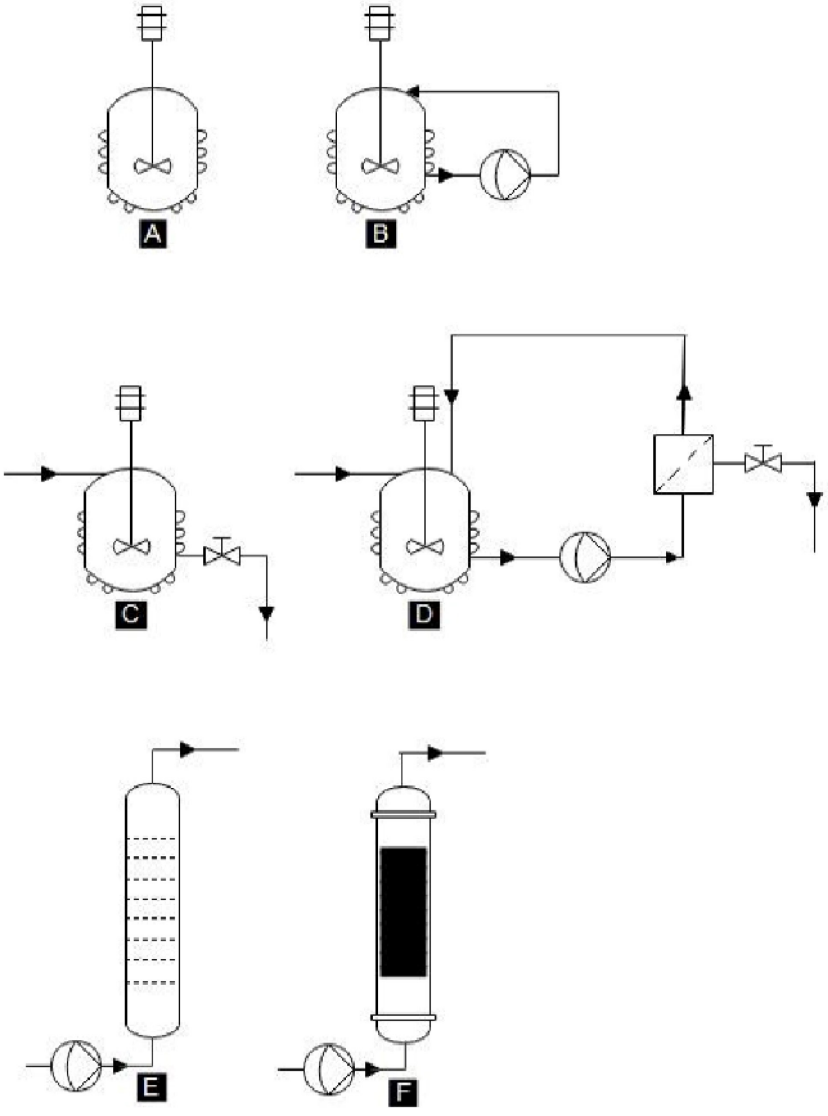

Fig. 3-2. Schematic reactor configurations for enzymes. Batch operation reactors: A) stirred tank; B) recirculation stirred tank. Continuous operation reactors: C) stirred tank; D) stirred tank membrane; E) packed bed; F) fluidized bed. (Adapted from [176]).

In batch mode operation, the biocatalyst is recovered after each batch process by centrifugation or filtration. If combined with a membrane filtration system, the biocatalyst can be directly recycled back to the batch reactor for a continuous operation. Despite this wide choice, there are very few studies on the continuous applications of lac-CLEAs. The trend in lac-CLEA-based technology focused on the development and stabilization of the biocatalysts (Table 3-2). These developments must ensure a high catalytic and operational stability to lac-CLEAs for an efficient continuous application. Nevertheless, few attempts to apply lac-CLEAs in continuous reactors have been proposed.

A perfusion basket reactor (PBR) was developed by [166] for the continuous operation of lac-CLEAs. The PBR consisted of an unbaffled basket made of a metallic filtration compartment filled with the biocatalyst and the reaction solution, which were continuously agitated (Fig. 3-3). The continuous elimination of some EDCs (nonylphenol, bisphenol A and triclosan) was monitored at an initial concentration of 5 mg/L for each EDC. The results showed that most of the EDCs were removed from the solution (> 85% removal) with a hydraulic retention time of 325 minutes. The performance of the PBR was stable over a week of continuous running [166].

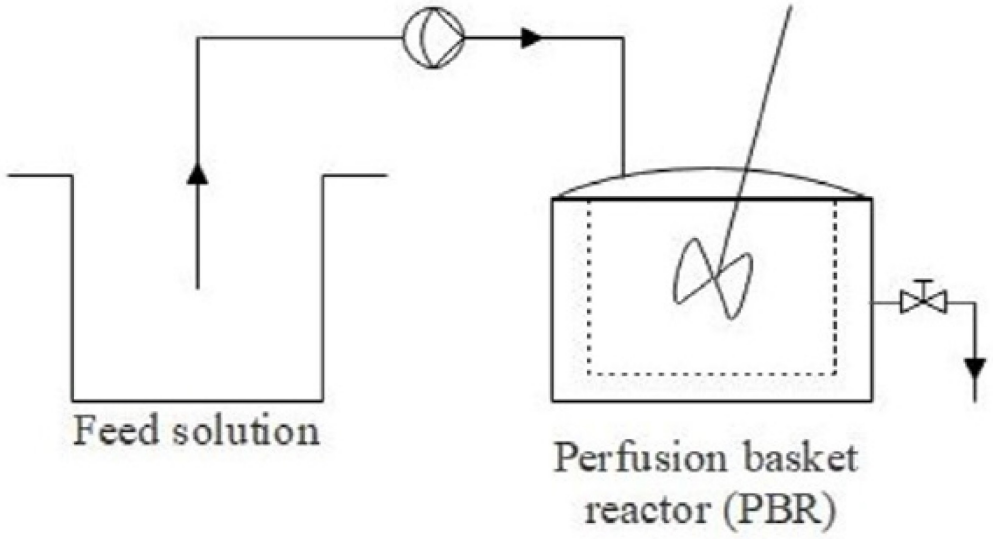

Fig. 3-3. Experimental setup of the perfusion basket reactor (adapted from [166]).

Another reactor system was proposed for the continuous application of lac-CLEAs. The developed Hybrid Bioreactor (HBR) proposed by Ba et al. 2014 consists of a stirred tank reactor containing lac-CLEAs, combined with a polysulfone hollow fiber microfiltration membrane [164]. The membrane filtration system confined the lac-CLEAs and recycled them back to the

reaction solution (Fig. 3-4). The HBR was operated continuously for pharmaceutical compounds (acetaminophen, mefenamic acid, carbamazepine) removal in aqueous solution at an initial concentration of 100 μg/L for each compound. The results showed that the synergistic effects of the biocatalyst and the membrane filtration achieved complete elimination of acetaminophen and mefenamic acid and over 90% elimination of carbamazepine from wastewater. In addition, the system efficiently recycled the lac-CLEAs during the experiment with negligible fouling membrane phenomena [164]. Later, the same HBR system was operated using a combi-CLEA made of laccase and tyrosinase for a continuous elimination of a mixture of 14 pharmaceutical compounds from municipal wastewater at an environmentally relevant concentration of 10 μg/L [33]. The results showed complete removal of all active compounds after five days of operation. After the treatment, the Combi-CLEA retained ~70% of its initial enzymatic activity.

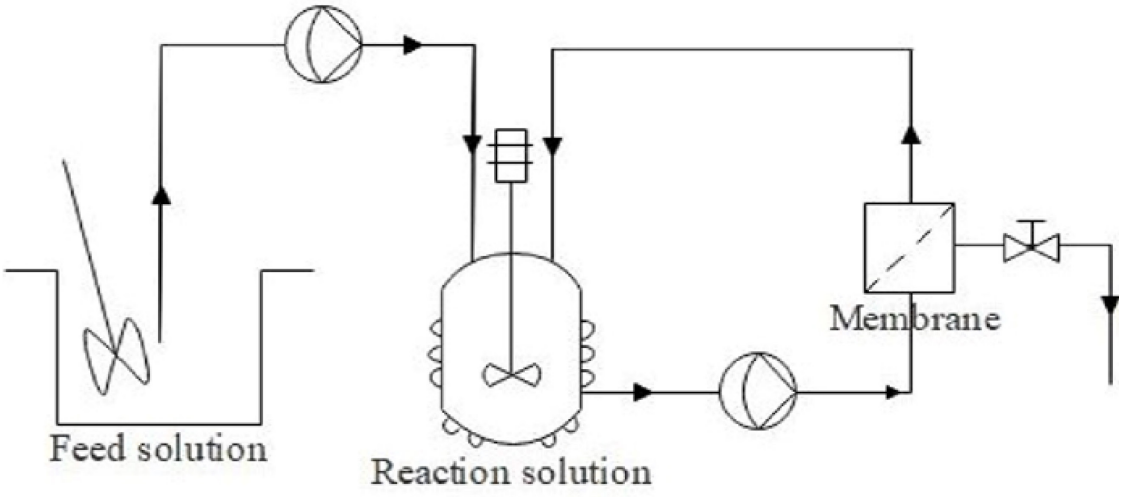

Fig. 3-4. Experimental setup of the hybrid membrane bioreactor (adapted from [164]).

These two examples demonstrated that the continuous operation of lac-CLEAs in reactors is feasible. However, the lack of a suitable design restricts the large-scale applications of lac-CLEAs. From these first attempts, it was shown that further investigation is needed to characterize the reactor design in depth and evaluate both parameters (catalytic and operational) for lac-CLEA applications in wastewater bioremediation.

The reactor design must be carefully chosen while taking into consideration the properties of the insolubilized biocatalyst. When applied to large-scale processes, lac-CLEAs will have low volumetric productivity which is influenced by the process volumetric flow. So, we believe that

for real application of lac-CLEAs in continuous mode, favored approaches, which are more compatible with potential streamflow, such as the one proposed by Ba et al. 2018 will be appropriate [33]. Another consideration when designing a suitable reactor for lac-CLEAs application is to understand and develop strategies to deal with the gradual decrease in enzyme activity during the operation processes [177].

All in all, the developed continuous reactors should be simple to implement for large-scale application purposes, adapted to current wastewater treatment plants and be economically sustainable [178].

3.8 Conclusions

Laccases are oxidoreductases, which show excellent potential for the elimination of emerging contaminants from municipal, industrial and hospital wastewaters. This potential has led to a focus on laccase immobilization and insolubilization techniques in recent decades. The insolubilization method using CLEA technology has been shown to apply to a wide range of enzymes, turning it into a useful tool for bioremediation processes. The simplicity of preparation, lower production costs from crude laccase extract and the possibility of combining laccases with other enzymes, are some of the advantages of CLEAs. Also, laccases only need oxygen to oxidize substrates, which is another significant economic advantage for large-scale bioremediation.

Recently, lac-CLEA technology has been improved with some strategies, such as a combination of formulation methods (e.g. physical entrapment of CLEAs) and the formation of polymer engineered structures. The developed strategies help to enhance the stability of lac-CLEAs and increase their applicability for wastewater bioremediation.

As the impact of PPCPs in the environment and the health risks associated with wastewater reuse are still not well-established, the growing interest in developing lac-CLEAs for wastewater bioremediation purposes gives hope for the refinement of current wastewater treatment. Lac-CLEAs may contribute to reducing the environmental risks associated with some PPCPs, which could help to ease and diversify wastewater reuse. At this time, efforts are still needed to understand the phenomenon governing the behavior and performance of lac-CLEA technology. In addition, to overcome the current limitations related to lac-CLEA operations in continuous

processes, more scaling-up experiments and applications in real conditions are needed. The choice of lac-CLEA technology for bioremediation of PPCPs from wastewater of other industrial biotechnological applications can be accelerated by the economic viability assessment of the biocatalyst production. To the best of our knowledge, no cost estimation of insolubilized lac-CLEA production has been published. Information on the profitability of lac-CLEA production could help industries and decision-makers analyze the feasibility of these sustainable technologies in bioremediation processes. Also, the lack of a suitable reactor design for the continuous application of insolubilized laccases also limits the lac-CLEA application in large-scale processes.

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Chapitre 4 - Prétraitement fongique d'un effluent industriel de bioraffinerie et évaluation concomitante de la production de laccase

4.1 Avant-propos

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Titre en français : Mycoremédiation de phénols et d'hydrocarbures aromatiques polycycliques dans un effluent de bioraffinerie et production concomitante d'enzymes ligninolytiques.

Contribution au document : Cet article propose une stratégie de sélection de WRFs pour la mycoremédiation de contaminants organiques persistants dans un effluent industriel de bioraffinerie. Cette étude a également permis de mettre en évidence la possibilité d'exploiter une stratégie de déphénolisation par mycoremédiation de l'effluent pour la production d'enzymes d'intérêt biotechnologique comme la laccase.

Résumé en français : Cette étude a évalué la capacité de sept souches fongiques à éliminer les phénols et à produire des enzymes à partir d'un effluent de bioraffinerie (BRW) à pH 7,0 non stérilisée résultant de la valorisation de la biomasse de bois traité. Deux stratégies consistant à ajouter du glucose et des copeaux de bois à faibles concentrations (4 g L^{-1}) pour supporter la croissance des champignons ont été étudiées. La caractérisation chimique du BRW a révélé la présence d'une concentration importante de certains composés organiques et inorganiques toxiques (chlorophénols, hydrocarbures aromatiques polycycliques et métaux lourds). Comparée à la biosorption, la biotransformation était le principal mécanisme impliqué dans l'élimination des contaminants. La mycoremédiation, utilisant *Pleurotus dryinus* et *Trametes hirsuta*, a atteint des taux de déphénolisation élevés (94 % et 100 %, respectivement), et a contribué à l'élimination d'autres composés organiques et inorganiques. Les résultats ont également démontré un potentiel de production d'enzymes ligninolytiques telle que la laccase durant le prétraitement du BRW. L'utilisation de déchets lignocellulosiques comme source de carbone a contribué à diminuer le coût du milieu de culture, mais pas le coût final de la production de la laccase. L'estimation des coûts de production de la laccase a montré que la stratégie 1, qui utilisait le milieu le plus cher (avec le glucose), était 60 fois et 4 fois moins chère pour *Pleurotus dryinus* et *Trametes hirsuta*, respectivement, que la stratégie 2, qui utilisait des copeaux de bois. La durée du traitement était un paramètre crucial à considérer pour obtenir une production rentable de laccase durant la mycoremédiation du BRW.

Mots-clés : Bois traité; effluent de bioraffinerie; Mycoremediation; Élimination des phénols; Hydrocarbures aromatiques polycycliques; Laccase; Estimation de coûts.

Title: Mycoremediation of phenols and polycyclic aromatic hydrocarbons from a biorefinery wastewater and concomitant production of lignin modifying enzymes.

Abstract: This study assessed the ability of seven fungal strains to remove phenols and produce enzymes from a non-sterilized pH 7.0 biorefinery wastewater (BRW) resulting from the valorization of treated wood biomass. Two strategies using glucose and woodchips at low concentrations (4 g L^{-1}) were studied. The chemical characterization of the BRW revealed the presence of a significant concentration of some toxic organic and inorganic compounds (chlorophenols, polycyclic aromatic hydrocarbons and heavy metals). Compared to biosorption, biotransformation was the primary mechanism involved in the elimination of the contaminants. Mycoremediation, using *Pleurotus dryinus* and *Trametes hirsuta,* achieved high dephenolization yields (94% and 100%, respectively), contributed to the removal of some other organic and inorganic compounds, and concomitantly produced laccase in the BRW. The use of lignocellulosic waste material as the carbon source contributed to decreasing the cost of the culture medium but not the final cost of laccase production. The laccase production cost estimate showed that Strategy 1, which used the most expensive medium (with glucose), was 60-fold and 4-fold less expensive for *Pleurotus dryinus* and *Trametes hirsuta*, respectively, than the Strategy 2, which used woodchips. The processing time was a crucial parameter to consider for achieving cost-effective of laccase production from sustainable BRW mycoremediation.

Keywords: Treated wood; Biorefinery wastewater; Mycoremediation; Phenol removal; Polycyclic aromatic hydrocarbon; Laccase; Cost estimate.

4.2 Introduction

Biorefineries have been gaining substantial attention because of their sustainability and the renewable nature of the biomass they use. The raw materials used in biorefineries can include treated wood used for utility poles, railway ties or sidewalks [179,180]. Canada is one of the primary producers of treated wood. The treated wood is obtained by the usage of pentachlorophenol (PCP), creosote and chromated copper arsenate (CCA) to protect the wood from the deterioration caused by microbial colonization [22]. Canadian treated wood is mostly used within the country, with only 10% of it being exported. Quebec is one of the largest wood

producers in the country, and as a result, biorefineries have gained more attention in this province [181].

Enormous opportunities exist for generating high-value products from recycling treated wood after its service life. However, the valorization process of treated wood releases harmful contaminants (chlorinated phenolic compounds, polycyclic aromatic hydrocarbons (PAHs), and heavy metals). Chlorophenols and PAHs are harmful to human health and can cause severe ecotoxicological issues due to their persistence and bioaccumulation properties. These compounds are known to accumulate in the liver, kidneys, adipose tissue and can be metabolized into even more toxic compounds. Their carcinogenic and mutagenic effects are also well established [3,48], and some of them are listed by the United States Environmental Protection Agency (USA-EPA) and the National Pollutant Release Inventory (NPRI) as priority environmental contaminants.

Consequently, due to the requirement of preliminary detoxification of the biomass, the recycling process of the treated wood can be challenging. The decontamination steps of the biomass generate toxic wastewater [182]. The discharge of this untreated wastewater into the environment or sewage network is not accepted as it contains high concentrations of hazardous chemicals from the wood preservatives.

Additionally, the high concentration of hazardous compounds does not allow conventional bioremediation treatment [20]. BRW from the lignocellulosic waste treatment processes can provide carbohydrates and other nutrients (i.e., organic and inorganic compounds), which can support microbial growth. However, microbial growth in biorefinery wastewater (BRW) to support biotechnological applications has not yet been investigated in depth. Microbes can grow in BRW and thus bring a promising alternative to exploit BRW as a resource, thereby helping to promote a sustainable treatment process. Furthermore, metals and lignin derivatives from wood material in BRW could support mycoremediation, by inducing fungal ligninolytic enzyme activity [20]. It is well known that fungi and their lignolytic enzymes can oxidize a wide range of chemical compounds in a variety of waste streams [10]. Thus, assessing the fungal ligninolytic capacity could be a valuable approach from a bioremediation perspective, to develop a sustainable mycoremediation process for BRW treatment.

Fungal growth in BRW, to support potential biotechnological applications (i.e., simultaneous pollutant removal and enzyme production), has not been investigated to any great extent to date. The mechanisms involved in fungi-based biotransformation processes include the extracellular production of lignin-modifying enzymes (LME) such as laccase (LAC) and lignin peroxidase (LIP), intracellular enzymes such as cytochrome P-450 and monooxygenase systems and sorption phenomena [14]. The efficiency of these enzymes for bioremediation has been extensively studied [95]. Although LME offers some advantages for environmental biotechnology applications, their utilization is still limited due to the relatively high production costs associated with using traditional growth media [183]. One potential solution for reducing the production cost of these enzymes could be using industrial waste streams as the growth media. Such an approach warrants further attention to determine whether these bioprocesses can be transferred to large-scale purposes, thereby appropriately addressing industrial needs.

In a previous study, twelve fungal strains from our in-house culture collection were screened to determine their potential to grow in BRW from treated wood. Seven fungal strains tolerated the toxic BRW conditions over a twelve-day incubation period [20]. The present work has been focused on these seven strains, intending to identify changes in the organic fraction of BRW during the mycoremediation process. Firstly, it was necessary to characterize the BRW. Secondly, the ability of the strains to produce LME for bioremediation of the BRW under the studied strategies was assessed. Finally, the mycoremediation potential based on the dephenolization of the BRW was evaluated, and a preliminary economic aspect of LAC production as a value-added product was assessed.

4.3 Materials and methods

All the reagents were of analytical grades and obtained from Fluka (Buchs, Switzerland) and Sigma-Aldrich (Saint-Louis, Missouri, United States). The experiment was performed under batch conditions in duplicate.

4.3.1 Characterization of the biorefinery wastewater

A second generation lignocellulosic biorefinery wastewater located in Quebec, Canada was used in this study. The total phenol content was evaluated spectrophotometrically. This method is based on the use of 4-aminoantipyrine (AAP) [184]. Briefly, phenolic compounds react with

AAP at pH 8 in the presence of potassium ferricyanide to form a colored antipyrine dye. Then the absorbance of the solution was measured at 500 nm. A calibration curve with phenol as the standard (in the range of 1 to 50 ppm) was used to calculate the total phenol concentration.

Phenolic compounds and PAHs were analyzed according to the standard method of the Quebec Centre of Expertise in Environmental Analysis [185,186]. A liquid/liquid (v/v) of the culture medium supernatant and solid-liquid extraction of the mycelium pellets for biosorption characterization was performed. Extractions were repeated twice using dichloromethane as solvents, as described by González-Abradelo et al. (2019) [187]. A G1800A Gas Chromatography-Mass Spectrometry (GC-MS) equipped with an electron ionization detector and an HP-5 MS fused-silica column (30cm × 0.25mm, 0.25mm film thickness) (Hewlett-Packard Company, Palo Alto, California, United States) was used to obtain an accurate quantification of phenols and PAHs. The data were acquired with the instrument operating in electronic impact ionization mode and selective ion acquisition mode. The injection volume was 1 µL. The limit of detection of the method was of the order of 0.05 µg L^{-1} and the quantification limit was 0.15 µg L^{-1}. The column was held at 40 ºC for 0.5 min then temperature was increased to 310 ºC for 5 min at the rate of 20 ºC min^{-1}, 2 ºC min^{-1} and 25 ºC min^{-1} at the respective programming levels of 120 ºC, 170 ºC and 310 ºC for phenolic compounds. For PAHs, the column temperature was held at 80 ºC for 1 min then temperature was increased to 335 ºC for 10 min at the rate of 35 ºC min^{-1} and 3 ºC min^{-1} at the respective programming levels of 320 ºC and 335 ºC. Helium was used as carrier gas at the flow rates of 1.1 ml min^{-1} and 1.4 ml min^{-1} respectively for phenolic compounds and PAHs. A total of twenty-eight phenolic compounds, fifteen PAHs and eight heavy metals were measured in the BRW. The concentrations of all analyzed compounds are given in Appendix A (Annexe 1).

Heavy metal concentrations were determined by using an inductively coupled plasma mass spectrometry (ICP-MS), equipped with an AS-93 auto-sampler (PerkinElmer Company, Woodbridge, Ontario, Canada). The method was set up as follows: 20 sweeps per reading, one reading per replication and three replications per sample. Germanium was used as an internal standard. A standard curve was prepared from 1,000 ppm single element ICP-MS grade standards (Delta Scientific, Mississauga, Ontario, Canada) and 2% nitric acid as the diluting solvent. The calibration was from 1 to 500 µg L^{-1}. Diluting solvent and wash solutions were

prepared using nitric acid and ultra-pure water (18.2 MΩ.cm^{-1}) (ELGA Purelab System, ELGA LLC, Canada).

The chemical oxygen demand (COD) of the wastewater was determined by the standard method using the Hach-DRB-200 analyzer [188]. The COD values were determined by using AccuSPEC COD reagent tubes (1,500 ppm) (SCP SCIENCE Company, Québec, Quebec, Canada). The dilutions were prepared before the COD determinations, with the BRW being diluted between five and ten times to fit the recommended range of the analysis test tubes.

4.3.2 Fungal inoculation in biorefinery wastewater

Four ascomycetes (*Aspergillus caesiellus, Emericellopsis* sp.*, Pseudogymnoascus* sp.*, Trichoderma atroviride*) and three basidiomycetes (*Pleurotus dryinus, Phanerochaete chrysosporium, Trametes hirsuta*), were used during this study.

A. caesiellus was isolated from sugarcane bagasse enriched with 2M NaCl [189]. The *Emericellopsis* and *Pseudogymnoascus* species were isolated from deep marine sponges [182]. The *T. atroviride* strain used in this study carries a recombinant clone that expresses the LAC gene from *Pycnoporus sanguineus* [77]. The basidiomycetes, *P. dryinus* IBB 903, *P. chrysosporium* ATCC 787 and *T. hirsuta* MTCC 1171 were obtained from the Institute of Biochemistry and Biotechnology in Georgia, the American Type Culture Collection Centre, and the Microbial Type Culture Collection Centre in India, respectively.

The strains were grown in Petri plates containing potato dextrose agar (dextrose: 20 g L^{-1}, agar: 15 g L^{-1} and potato starch: 4 g L^{-1}) under sterile conditions. A 1 cm^2 agar plug from the fungal growing region was inoculated onto agar Petri dishes and incubated at 30 $^{\circ}$C for seven days to obtain the inoculum. After seven days of growth on agar plates, four seven-millimeter diameter plugs (selected at the same physiological state on the youngest border of one colony ≈ 10^8 spores) were separately inoculated into 100 mL of non-sterilized BRW in 250 mL Erlenmeyer flasks. The cultures were grown in batch conditions in an orbital shaker at 150 rpm at 27 $^{\circ}$C. The pH of the BRW was lowered to a neutral pH using 0.1 N sulfuric acid before inoculation.

Two strategies were assessed for mycoremediation of the BRW: *Strategy 1)* BRW supplemented with 4 g L^{-1} of glucose and *Strategy 2)* BRW supplemented with 4 g L^{-1} of cylindrical woodchips (6 mm in diameter and 10 mm in length) from *Pinus arizonica*. The sample was periodically

withdrawn to determine pH, protein concentrations and enzymatic activities: LAC, LIP and aryl alcohol oxidase (AAO), as well as phenols and PAHs measurement. The supernatants and pellets were obtained by centrifugation of the sample at 10,000 ×*g* for 15 minutes. Non-inoculated flasks and non-sterile BRW were used as controls. All the parameters mentioned above were determined for cultures derived from both strategies for the seven tested fungi.

4.3.3 Mycoremediation of the biorefinery wastewater

Protein concentration was estimated using the bicinchoninic acid (BCA) test kit from Thermo Fisher Scientific (Rockford, Illinois, United States). BCA determination was performed by colorimetric detection at 562 nm, and bovine serum albumin was used as a standard for total protein quantification [190].

LAC, LIP and AAO activities were determined according to previously reported methods [139]. LAC activity was determined by measuring the conversion of 1 mM 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to its radical cation ($ABTS^{\bullet+}$) at 420 nm (molar extinction coefficient (Ɛ) = 36,000 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer (pH 4). The LIP assay measured the oxidation of 2 mM veratryl alcohol at 310 nm (Ɛ = 9,300 M^{-1} cm^{-1}) in 0.1 M tartrate buffer at pH 3 in the presence of 0.4 mM H_2O_2. AAO activity was measured by following the oxidation of 10 mM veratryl alcohol to veratraldehyde at 310 nm in 0.1 M sodium phosphate buffer, pH 6.

All protein content and enzyme activity assays were conducted in 96-well plates and spectrophotometrically determined by using a double-beam UV – Vis spectrophotometer SpectraMax Plus 384 (Molecular Devices Corp., Sunnyvale, California, United States). One unit of enzymatic activity (U) represents the amount of enzyme that forms 1 µmol of product per minute.

The removal of pollutants from the BRW was monitored after 38 days following fungal inoculation. Total removal and adsorption percentages were determined for both strategies. The extent of removal was calculated according to equation 1 and expressed as a percentage. $C_{initial}$ represents the initial phenol concentration in the BRW before treatment and C_{final}, the final phenol concentration in the BRW after treatment.

$$Extent\ of\ removal = \left((C_{initial} - C_{final})/C_{initial}\right) \times 100 \quad (1)$$

The adsorption capacity was determined by washing the pellets with sterile deionized water and vigorously shaking and sonicating them after centrifugation to facilitate the desorption. The most promising strains, according to the dephenolization ability and LME production, were selected for subsequent experiments.

4.3.4 Cost Estimate of laccase production from biorefinery wastewater mycoremediation

The production of value-added products from a mycoremediation treatment is a sustainable alternative to valorize this treatment by the production of valuable products such as enzymes, polysaccharides or other organic compounds [191]. In this study, instead of considering the BRW and fungal biomass as waste to discard, we considered them as valuable products to produce a potentially high biotechnological enzyme (LAC).

The cost estimate of LAC production helps to determine whether the application of a non-sterilized bioprocess strategy of utilizing the BRW to produce an enzyme can benefit the overall mycoremediation process. All prices are given in 2019 Canadian dollars, CAD ($), and all the costs were evaluated for one cycle of 18 litres of BRW mycoremediation, based on the shaker capacity. The calculations are based on the cost estimate of biocatalyst production determined by Osma et al. (2011) [183]. Five terms contributing to the total cost of enzyme production were considered: the cost of the culture medium (C_{CM}), the equipment cost (C_{EQ}), the operation cost (C_{OP}), the time taken to obtain maximum enzyme activity (D_{MAX}) and the volume of the crude enzyme (V_{CE}).

For the C_{CM}, the production cost of BRW was considered insignificant because it is a waste that can be disposed of. For this reason, its cost was assumed to be null. However, we considered the cost of the sulfuric acid used to adjust the pH of the BRW ($0.004 per production cycle) based on a review of the sulfuric acid market in 2017 [192]. To help fungal growth and enzyme production, BRW was enriched with two carbon sources; glucose ($0.026 per production cycle) and woodchips ($0.004 per production cycle) at low tested concentration (4 g L^{-1}) for both strategies. The market price for glucose was $0.360 kg^{-1} [193], and $0.060 kg^{-1} of dry weight based on local sawmill prices in February 2019 for the woodchips.

Regarding the C_{EQ}, two equipment models were used for enzyme production, a Megafuge 8 centrifuge from Thermo Fisher Scientific (Montreal, Quebec, Canada) and an Innova 2300 platform shaker from New Brunswick Scientific (Midland, Ontario, Canada). Each C_{EQ} was expressed based on the price of the equipment, its capacity and lifetime (Equation (2)). Let us assume that D_{MAX}, the maximum enzyme activity of the culture, was obtained in 30 days. Then, the number of cycles that the given equipment can be used is 12 times every year. The capacity of the equipment (C_{AP}) has been taken into consideration to determine the maximum number of cultures that can occur in each cycle. The manufacturer's description was consulted to calculate the power consumption and capacity of the equipment. Another essential term in this process is the lifetime (LT) of the given equipment, which is the warranty period. The warranty periods were: 730 days for the shaker and 365 days for the centrifuge. The price (P) of the shaker was $9,611, and the price (P) of the centrifuge was $2,139.

$$C_{EQ} = P/\left((365/D_{MAX}) \times LT \times C_{AP}\right) \quad (2)$$

The total C_{EQ} is represented as the sum of the C_{EQ} of the shaker and the centrifuge (equation 3).

$$Total\ C_{EQ} = \left(C_{EQ}\ centrifuge + C_{EQ}\ shaker\right) \quad (3)$$

The energy spent using the shaker and the centrifuge reflects the culture conditions in the calculation and is considered as the C_{OP}. The human resource cost of the given process was not considered supposing that the process is automated and does not require manual labor. The operating capacity of the centrifuge is limited by the capacity of the shaker. So, the C_{EQ} and C_{OP} of the centrifuge were calculated using the C_{AP} of the shaker (equations 2 and 4).

$$C_{OP} = (E \times D_{MAX} \times 24)/C_{AP} \quad (4)$$

E represents the energy price in CAD ($). Its calculation was obtained by equation 5. Operation time for the shaker was considered equal to D_{MAX} and was estimated at 15 hours for the centrifuge.

$$E = Energy\ price\ (\$\ kW^{-1}h^{-1}) \times Power(kW) \times Operation\ time(h) \quad (5)$$

The total C_{OP} is represented as the sum of the C_{OP} of the centrifuge and shaker (equation 6).

$$Total\ C_{OP} = (C_{OP}\ centrifuge + C_{OP}\ shaker) \quad (6)$$

The total enzyme production cost (C_T) is affected by C_{CM}, C_{EQ} and C_{OP} (equation 7).

$$C_T = \left(C_{CM} + Total\ C_{EQ} + Total\ C_{OP}\right) \quad (7)$$

The final enzyme production cost (C_{EP}) is the measure of the total enzyme production cost divided by the product of maximum enzyme activity (D_{MAX}) and volume of crude extract (V_{CE}) that was fixed as the capacity of the shaker (18 litres) (equation 8).

$$C_{EP} = Total\ cost/(D_{MAX} \times V_{CE}) \quad (8)$$

4.3.5 Statistical analysis

Statistical analysis was performed by variance analysis (one-way ANOVA) using Tukey test to determine statistically significant differences. The analysis tools of SigmaPlot version 12.0 (Systat Software, San Jose, CA) was used for the statistical analysis. All the assays were carried out in triplicate with three independent measurements for each test, and the mean value was used (± standard deviation).

The levels of significance are expressed as a p-value < 0.05. All analysis was performed in triplicate.

4.4 Results and discussion

4.4.1 Initial characteristics of biorefinery wastewater

Two groups of organic pollutants (phenolic compounds and PAHs) were identified and quantified in the BRW (Annexe 1: Appendix A). The BRW displayed a high COD level (3.5 g L^{-1}). The phenols, in their chlorinated forms, were predominant. Twenty-eight phenolic compounds were identified with a total concentration of 28,323 µg L^{-1}. PCP, the most commonly used wood preservative was predominant at 84%, followed by 2,3,5,6-tetrachlorophenol (TCP), which represented 15.5% of the total phenolic compound's concentration (Table 1). Fifteen PAHs were measured in the BRW, with a total concentration of 498.5 µg L^{-1}. The main PAHs detected were phenanthrene (PHE), fluoranthene (FLU), pyrene (PYR) and benzo(a)anthracene (BAA) (Table 1).

Heavy metals were also found in the BRW. The major compounds were arsenic (2,950.0 μg L^{-1}) followed by copper (827 μg L^{-1}), iron (709 μg L^{-1}) and zinc (436 μg L^{-1}). Other metals were found at lower concentrations. These are: manganese (61 μg L^{-1}), mercury (28 μg L^{-1}), lead (19 μg L^{-1}) and cadmium (1 μg L^{-1}) (Annexe 1: Appendix A).

Copper, iron, manganese and zinc are essential metals for living organisms. However, they are toxic at high concentrations. Other metals such as cadmium, mercury and lead are also known to display toxic properties [194]. Arsenic is also a toxic metal found in the environment and has demonstrated carcinogenic effects in humans, as well as toxicity in plants and animals [195].

BAA is classified as a potential human carcinogen, while the carcinogenic potential of FLU, PHE and PYR has not yet been established. The Canadian Environmental Quality Guidelines (CEQG) provides recommendations for water quality and terrestrial ecosystems regarding the concentration of phenols and PAHs in the environment. The concentration of phenols and PAHs present in the BRW was very high when compared to the guidelines (Table 4-1).

The characterization of the BRW shows that the effluent contains a cocktail of toxic compounds. So, it is necessary to find an appropriate pretreatment before discharging it into a sewage treatment plant or the environment.

Table 4-1. The physicochemical parameters and concentrations of selected phenols [pentachlorophenol (PCP) and 2,3,4,6-tetrachlorophénol (TCP)], and polycyclic aromatic hydrocarbons (PAHs) [benzo(a)anthracene (BAA), phenanthrene (PHE), fluoranthene (FLU) and pyrene (PYR)]. Cs represents the aqueous solubility at 25 degrees Celsius and Log Kow, the octanol-water partition coefficient.

	Compound	Molar mass ($g\ mol^{-1}$)	Cs (25 °C) ($mg\ L^{-1}$)	Log Kow	Concentration in BRW ($\mu g\ L^{-1}$)	Recommendations [a] CEQG ($\mu g\ L^{-1}$) * ($mg\ kg^{-1}$ DW) #	Health and environmental risks [b]
Phenols	PCP	266.3	14	5.1	23,800 ± 2,380	0.5* 7.6#	Carcinogenic Very toxic to aquatic organisms
	TCP	231.9	1×10^{3}	4.4	4,380 ± 526	n.d.* 0.5#	
PAHs	BAA	228.3	9.4×10^{-3}	5.7	18.8 ± 2	0.018* n.d.#	Carcinogenic, teratogenic toxic to aquatic organisms
	FLU	202.3	0.26	5.1	121.4 ± 14	0.04* n.d.#	Toxic to some organs Toxic to aquatic organisms
	PHE	178.2	1	4.4	212 ± 24	0.4* n.d.#	
	PYR	202.3	0.13	4.8	86.8 ± 10	0.025* n.d.#	Toxic and bioaccumulation effect in fish, molluscs and algae

[a] Canadian Environmental Quality Guidelines for *pure water quality and aquatic life protection, and #for soils and human health for the residential and park environment.

[b] Data from PubChem – https://pubchem.ncbi.nlm.nih.gov/compound and International Agency for Research on Cancer – http://www.cancer-environement.fr/226-Classification-du-CIRC.ce.as

n.d.: data not available.

4.4.2 Fungal growth in biorefinery wastewater

Preliminary experiments have demonstrated that any of the studied fungi could grow in the hyperalkaline BRW at pH 12.4. In this study, the pH of the BRW was adjusted to neutral pH 7.0 using sulfuric acid before inoculation. The common trend observed when the fungi were grown in BRW supplemented with glucose was a shift in the pH to an acidic range ($4 < pH > 5$). In contrast, when the BRW was supplemented with woodchips, the pH increased to an alkaline range ($8 < pH > 9$).

pH plays a significant role in mycoremediation because fungal growth, enzymatic activity and stability, and the contaminants' availability are pH-dependent [196]. When the fungi were grown in the presence of sugars, it resulted in the production of organic acids, which contributed to the acidification of the media [197]. The pH value is also a controlled parameter for industrial wastewater before it can be discharged into the municipal sewage network. The variation in pH, which occurred during fungal growth when supplemented with woodchips, would be acceptable for the discharge of the effluent after mycoremediation. However, the BRW treated in the presence of glucose would require an additional pH adjustment step, before being discharged, which would potentially increase the overall operational costs.

The initial protein concentration in the BRW was determined to be 2 g L^{-1}. For both strategies, and all the fungal strains, the dynamics of protein production were similar. In general, during the first week (Day 0 to Day 6), the protein content increased to reach a maximum concentration of 3 to 4 g L^{-1}, before it slightly decreased and stay constant between 2 and 3 g L^{-1} (data not shown). The control samples displayed an insignificant variation in protein concentration over the experiment. So, the most significant contribution to the increase in protein was not due to the original microbiota, but, to the fungal growth and LME production in BRW.

The most studied LME in biotechnological applications is LAC. Maximal LAC activity was observed in *P. dryinus* and *T. hirsuta* after Day 7 and Day 3 when supplemented with glucose and after Day 35 and Day 3 in the presence of woodchips, respectively, for both strains. No LAC activity was observed after Day 20 for *T. hirsuta* for both strategies, contrary to *P. dryinus,* which displayed LAC activity throughout the experiment. Other enzymes among the tested LME were also detected during the fungal growth. All the fungal strains produced AAO when

BRW was supplemented with glucose. *T. hirsuta* and *Emericellopsis* sp. showed the highest AAO activity on Day 16 and Day 13, respectively (Table 4-2). When BRW was enriched with woodchips, only three basidiomycetes (*P. chrysosporium, P. dryinus* and *T. hirsuta*) produced AAO on Day 9. LIP activity was only observed in *T. hirsuta* on Day 3 when it was inoculated in the presence of glucose. In BRW supplemented with woodchips, LIP activity was observed on Day 6 in two ascomycetes (*A. caesiellus* and *Emericellopsis* sp.), and the two basidiomycetes (*P. dryinus* and *T. hirsuta)* on Day 6 and Day 3, respectively (Table 4-2).

Table 4-2. Laccase (LAC); lignin peroxidase (LIP); aryl alcohol oxidase (AAO) maximum activity detected in the BRW.

Fermentation system	Enzymes	Fungal strain	Maximum enzyme production	
			Enzyme activity (U L^{-1})	Culture duration (days)
Strategy 1 (Glucose)	AAO	*A. caesiellus*	70	16
		Emericellopsis sp	107	17
		Pseudogymnoascus sp.	63	16
		T. atroviride	63	9
		P. chrysosporium	81	13
		P. dryinus	75	16
		T. hirsuta	119	16
	LAC	*P. dryinus*	32	7
		T. hirsuta	17	3
	LiP	*T. hirsuta*	139	3
Strategy 2 (Woodchips)	AAO	*P. chrysosporium*	69	10
		P. dryinus	65	13
		T. hirsuta	84	9
	LAC	*P. dryinus*	60	3
		T. hirsuta	47	35
	LiP	*Emericellopsis* sp	89	16
		A. caesiellus	136	3
		P. dryinus	59	6
		T. hirsuta	131	6

The nature and concentration of the carbon source are known to play a critical role in LME production in fungi [198]. Thus, the BRW or the added carbon sources (glucose or woodchips) appeared to have an influence on LME production during fungal growth. Glucose at concentrations ranging from 5 to 20 g L^{-1} has been used as a co-substrate to enhance fungal growth in bioremediation processes [199,200]. For industrial applications, it could be

excessively expensive, making the process economically unsustainable. However, some other waste materials could be used to replace glucose. BRW is a very complex matrix that contains many pollutants with unknown cocktail effects on microbial growth. Therefore, the effects of adding glucose at low concentration (Strategy 1) and woodchips (Strategy 2) to the BRW as grow medium was assessed.

In this work, the tested strategies demonstrated that, as a cheap and effective culture medium, BRW supplemented with lignocellulosic waste material could support both fungal growth and LME production for mycoremediation.

4.4.3 Fungal screening based on biorefinery wastewater dephenolization

Practically all fungi caused a high total phenol removal rate (>85%) for both strategies after 30 days, apart from *Emericellopsis sp.,* which showed a lower removal rate (51%) when supplemented with woodchips (Fig. 4-1). The biosorption potential of all the strains was around 10%, except for *A. caesiellus* and *P. dryinus,* where biosorption of phenols was not observed. *T. hirsuta* also showed an insignificant sorption potential (4 % and 6 % for Strategy 1 and Strategy 2, respectively) (Fig. 4-1).

The total phenol removal rate was slightly lower when BRW was enriched with woodchips than with glucose. When BRW was supplemented with woodchips, the maximal phenol concentration observed during the experiment was twice the initial concentration in the first two weeks of treatment. After Day 15, the phenol content gradually decreased until the end of the experiment (time course data not shown).

Mycoremediation efficiency of phenolic compounds in aqueous streams is a function of various parameters such as temperature, substrate concentration, enzyme type, pH, reaction time or rotational speed [83]. LAC has been recognized to be an efficient enzyme for phenol removal [201]. The basidiomycetes that showed LAC activity (*P. dryinus* and *T. hirsuta*) were the most efficient strains for phenol degradation. However, LAC may not be the only enzyme involved in the BRW dephenolization process. These results also indicate that phenol removal in BRW was predominantly attributed to fungal degradation and not to biosorption, excluding *Emericellopsis sp.* when supplemented with woodchips for which the total phenol removal (51 %) was caused by biosorption (49 %) (Fig. 4-1).

Comparable results in the removal of phenols from industrial wastewater using fungal treatment was observed under similar conditions (high phenol concentrations) [19,202]. So, among the forty-nine white-rot fungal strains screened for diluted olive-mill wastewater dephenolization, Ntougias et al. 2015, found that all strains were able to grow in the effluent and degrade more than 60% of total phenolic compounds after five weeks of incubation. *Pleurotus* and *Trametes* species were top degraders of phenol with respectively, more than 95% and 80% removal [19]. Basidiomycetes are more often used in fungal whole-cell treatment for phenol removal [203]. Our results are in accordance with previously reported works and show that basidiomycetes are more efficient in removing phenols (Fig. 4-1).

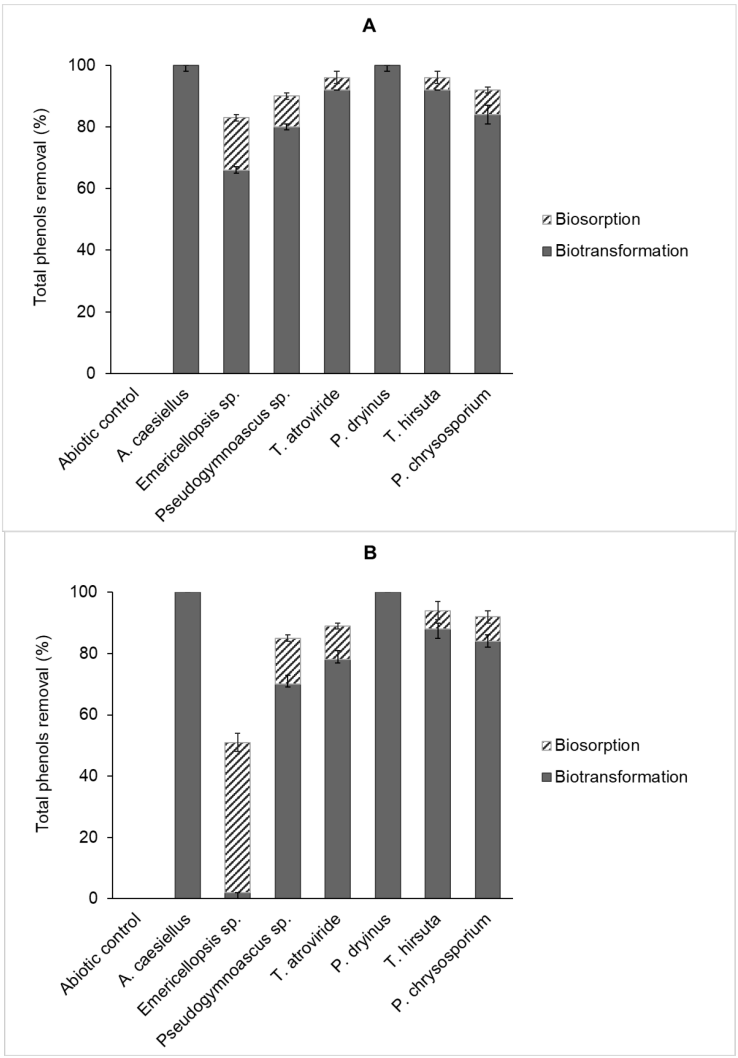

Fig. 4-1. Total phenol removal from the BRW by the studied strains (A: Strategy 1 and B: Strategy 2). Values plotted are the means and the error bars represent the standard deviation of triplicate assays.

The experimental conditions (dilution or sterilization), also play an essential role in phenol degradation processes. The sterilization process slightly increases the phenol degradation efficiency compared to non-sterile and diluted industrial olive mill effluent using *P. ostreatus* [202]. However, for an industrial application, such pretreatment of the effluent before mycoremediation would increase the processing cost. In this study, we showed that fungi can use phenolic compounds as a carbon source to grow under the tested non-sterile condition. So, such thermal pretreatment is not required for total phenol elimination from BRW.

Similar results have been published where the fungus *A. awamori* was able to remove 85% phenol content after six days in a liquid medium supplemented with phenolic compounds (3 g L^{-1}) as the only carbon and energy source [204]. These results open the door to a less expensive treatment option for industrial effluent rich in phenolic compounds. However, most of the time, bioremediation applied to real effluents, requires the addition of a co-substrate such as glucose. The co-substrate may help to increase the efficiency of the treatment, but it is usually considered as a drawback for the treatment because it will induce additional costs [205].

The depolymerization of lignin from the wood material increased the phenol level in the BRW. In some cases, using an inexpensive woodchip material as an external carbon source can be a source of secondary contamination that can slow down phenol removal from the BRW. Thus, screening different carbon sources and optimizing the concentrations is necessary.

4.4.4 Mycoremediation of organic and inorganic compounds from biorefinery wastewater

Fungi transform organic and inorganic compounds with their enzymes and synthesis system metabolites that perform physicochemical modifications. The variation of the organic compounds (Chlorophenols and PAHs) was monitored after 30 days culture for *P. dryinus* and *T. hirsuta* in BRW.

The elimination of phenolic compounds (PCP and TCP) was favoured in the presence of glucose (Fig. 4-2). The highest PCP removal (25.6 %) was observed in the presence of glucose for *T. hirsuta*. In contrast to phenolic compounds, a high PAHs removal level was observed, which reached up to 90% in some cases (Fig. 4-2). *P. dryinus*, demonstrated better PAHs removal potential than *T. hirsuta* when grown in presence of glucose, while both strains showed similar

removal levels when grown in BRW supplemented with woodchips (Fig. 4-2). Relative to the control samples, degradation of all the monitored organic contaminants was observed apart from PCP. In general, for both strategies, total removal percentages increased following inoculation of BRW with *P. dryinus* and *T. hirsuta* (Fig. 4-2).

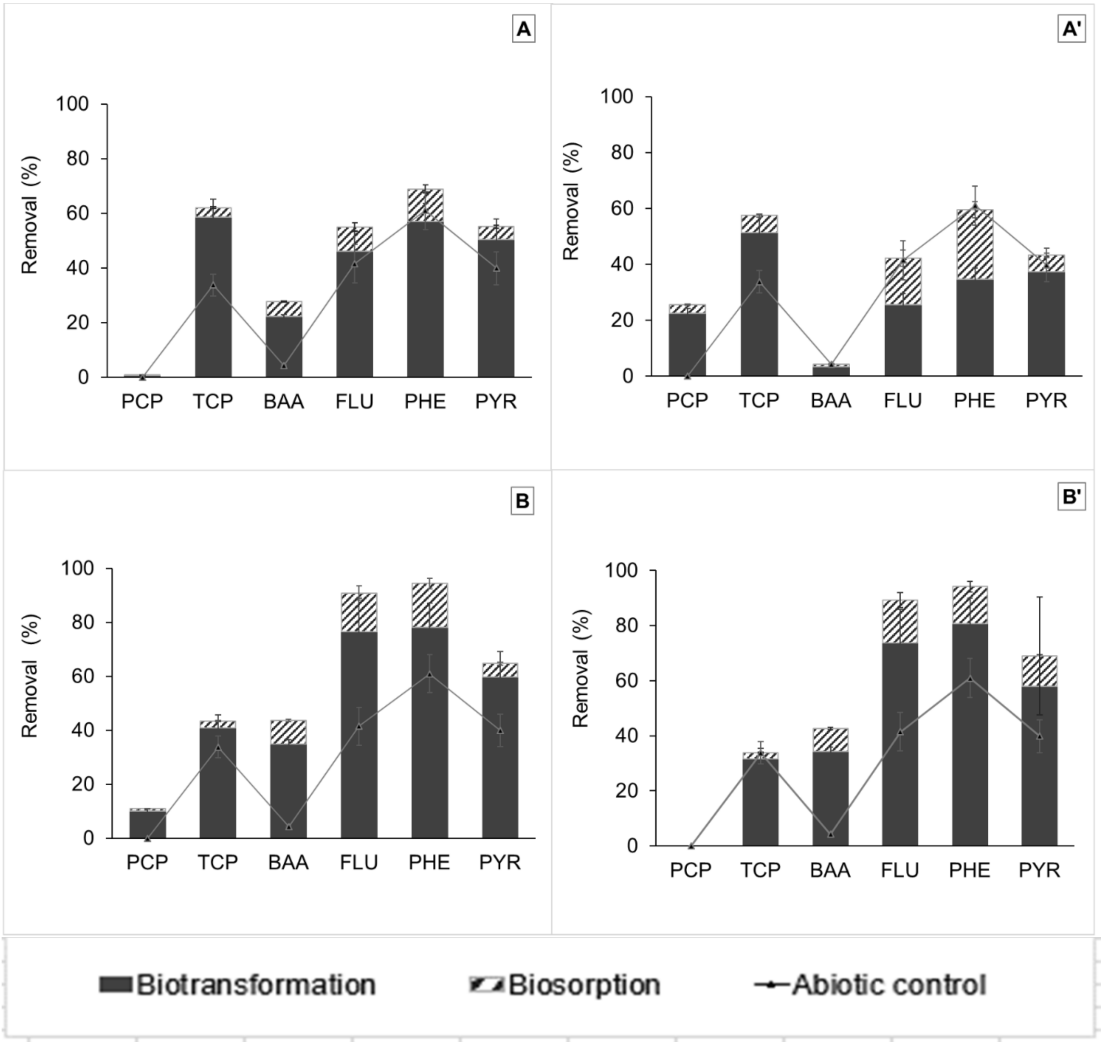

Fig. 4-2. Phenolic compounds and polycyclic aromatic hydrocarbons (PAHs) removal from BRW after mycoremediation treatment (A and A': represent respectively *P. dryinus* and *T. hirsuta* for Strategy 1, and B and B' represent respectively *P. dryinus* and *T. hirsuta* for Strategy 2). Values plotted are the means and the error bar*s represent* the standard deviation of triplicate *assays*.

The COD value allowed to determine any potential reduction in the degree of total organic composition in the BRW. The COD concentration decreased by around 30% for Strategy 1 and

20% for Strategy 2, which is quite encouraging. Regarding inorganic compounds, all metal concentrations found in the BRW complied with the legislation except for arsenic for which the concentration (2,900 μg L^{-1}) was almost three times above the regulation (1,000 μg L^{-1}). So, metal removal was not considered as a principal goal in this study. Only arsenic was a problematic heavy metal in BRW. The highest percentage of arsenic removal was obtained with Strategy 1 for both strains, with around a 10% removal of arsenic from the BRW, while Strategy 2 showed an insignificant increase from 1 to 3% in the arsenic level in the BRW. Our results indicate that acidic pH (Strategy 1) was most suitable for arsenic removal (Fig. 4-3).

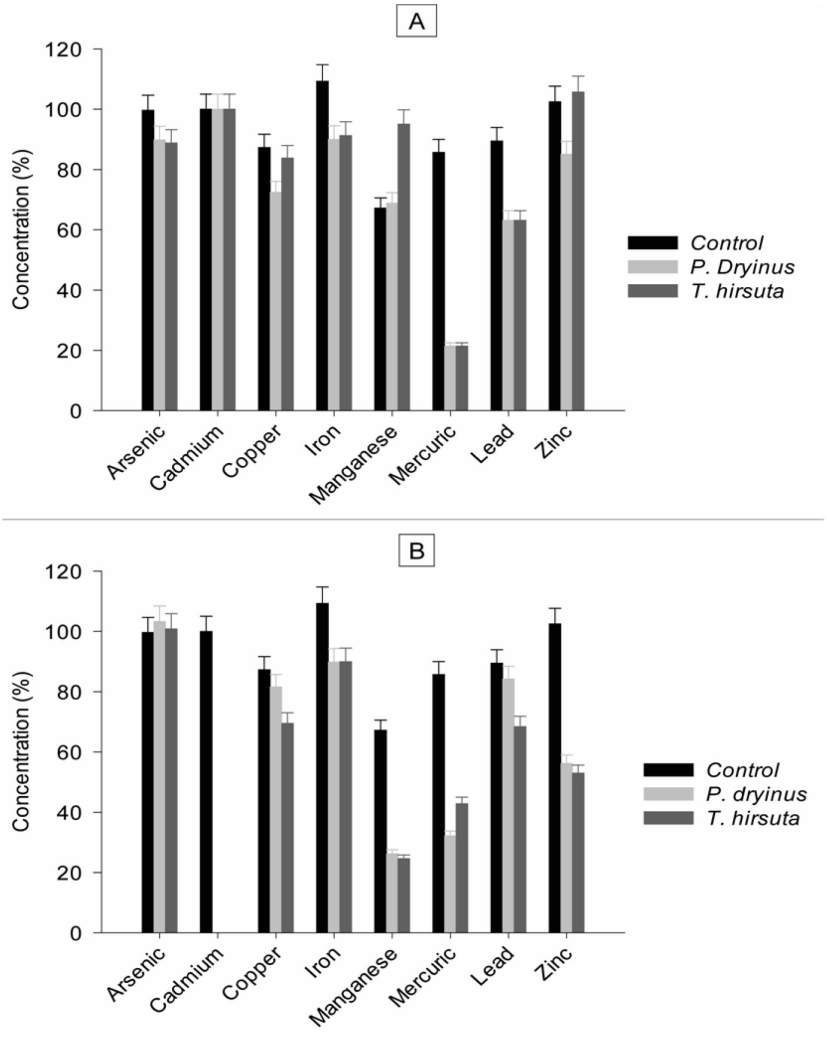

Fig. 4-3. Metal concentrations in the BRW before and after fungal treatments (A: Strategy 1 and B: Strategy 2). Values plotted are the means and the error bars represent the standard deviation of triplicate assays.

Chlorophenols are resistant to biodegradation, mainly when the chlorine atoms are located at both the *meta* and *para* positions [206]. For both strategies, the removal of PCP was challenging compared to TCP. PCP is a *para* and *meta*-substituted phenolic compound, which confers it a high degree of stability and limited biodegradability. Most of the PAHs present in the BRW are low molar mass compounds, which are known to be more readily biodegradable. The biodegradation of PAHs can be negatively impacted due to their low solubility, thereby limiting the application of liquid bioremediation processes [207]. Also, the presence of other compounds, such as heavy metals is known to facilitate PAHs removal [208].

In most cases, adsorption by the fungal biomass did not markedly contribute to the total removal. These removal levels again support the theory that the microbes present in the BRW are physiologically active. Thus, biotransformation was the primary mechanism involved in the elimination of phenols and PAHs.

However, like all monitored contaminants, COD reduction cannot be correlated to any specific enzyme activity, as a cocktail of ligninolytic enzymes and intracellular degradation mechanisms are likely to be involved in the mycoremediation process [14]. The COD removal levels observed were lower than those observed in the literature (> 40%) for the removal of organic compounds [19]. It is important to remember that the experiment described here was not conducted under optimal growth conditions, which might improve the removal of xenobiotics.

The removal of arsenic in water depends on parameters such as temperature, pH and organic compounds. These results are consistent with [209] study, which demonstrated that the arsenic removal percentage decreased as the pH of the solution increased. Arsenic can be removed from a solution by biotic absorption, adsorption of iron or manganese hydroxides or by a fixation with organic matter [210]. All these interactions can influence the presence of arsenic in the BRW. Fungi are also using some pathways to entrap and accumulate metals in their cells. Therefore, the slight increase in arsenic concentration in Strategy 2 could also be due to cells lysis [209]. Despite the differing results from Strategies 1 and 2, additional experiments are required to optimize and better understand the mechanisms of *P. dryinus* and *T. hirsuta* in arsenic removal from complex BRW.

Mycoremediation using *P. dryinus* and *T. hirsuta* achieved high dephenolization yields and contributed to removing some other organic and inorganic compounds, and concomitantly

producing LME from a hazardous BRW in non-sterile condition. As bioremediation depends a lot on the process operation conditions, further studies are required for better characterization of the behavior of these strains in BRW mycoremediation. Efforts are needed to focus on optimization of the physicochemical parameters (pH, carbon source type and concentration), considering the variability in the effluent composition and the large-scale implementation.

4.4.5 Economic assessment of laccase production

In the past decades, LAC from white-rot fungi (WRF) has found a prominent place in various biotechnological applications, and it is essential to find the cheapest ways to produce LAC. The use of waste materials as a substrate is economically advantageous, and cost analysis of this process is necessary to help industries choose the best treatment strategy. The cost estimate of this mycoremediation process is based on the work of [183] (see details in section 4.2.5). Among the tested fungi, *P. dryinus*, and *T. hirsuta* can be regarded as the best candidates to grow in BRW and to produce LAC. To produce the fungal culture independent of variants, like the cost of materials and nutrients used to prepare traditional media, BRW was enriched in a low concentration of glucose and an inexpensive readily available material (woodchips).

When supplemented with glucose, the C_{CM} ($0.30) is 30-times higher than the C_{CM} ($0.01) with woodchips (Table 4-3). This result confirmed that lignocellulosic waste material, as the main constituent of the fungal growth medium, results in a decrease in the C_{CM} [183]. The most sensitive parameter for such a fermentation process cost estimate is the D_{MAX}. A higher D_{MAX} implies a higher C_{EQ} and C_{OP}. For a D_{MAX} 11-times higher, LAC produced by *P. dryinus* in Strategy 2 showed C_{EQ} and $C_{OP,}$ respectively 8-times and 128-times higher compared with Strategy 1 (Table 4-3). The same trend was observed for *T. hirsuta* with a D_{MAX} 3-times higher, resulting in the C_{EQ} and the C_{OP} being respectively 3-times and 9-times higher in Strategy 1 (Table 4-3).

Table 4-3. Cost analysis of one cycle of laccase (LAC) production (18 Liters) under different cultivation conditions using biorefinery wastewater (BRW). The cost of culture medium (C_{CM}), total equipment costs (C_{EQ}), total operating costs (C_{OP}) and the final costs are given in Canadian dollars, CAD ($) for 2018-2019.

Fermentation system	Strategy 1				Strategy 2			
Fungal strains	*P. dryinus*		*T. hirsuta*		*P. dryinus*		*T. hirsuta*	
Equipment	Shaker	Centrifuge	Shaker	Centrifuge	Shaker	Centrifuge	Shaker	Centrifuge
Price P ($)	9,611	2,139	9,611	2,139	9,611	2,139	9,611	2,139
Lifetime LT (days)	730	365	730	365	730	365	730	365
Capacity Cap (L)	18	0.2	18	0.2	18	0.2	18	0.2
Operation time (h)	72	15	72	15	816	15	216	15
Power (kW)	1.2	0.31	1.2	0.31	1.2	0.31	1.2	0.31
Energy price ($ kW^{-1} h^{-1})	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
D_{MAX} (days)	3	3	3	3	34	34	9	9
LAC (U L^{-1})	31.7	31.7	16.9	16.9	60.8	60.8	38.4	38.4
C_{EQ} ($)	0.06	0.06	0.06	0.06	0.06	0.74	0.06	0.19
C_{OP} ($)	20.4	0.01	20.4	0.02	2,623.5	0.02	183.8	0.02
Total C_{CM} ($)	0.3		0.3		0.01		0.01	
Total C_{EQ} ($)	0.1		0,1		0.8		0.3	
Total C_{OP} ($)	20.4		20.4		2,623.5		183.8	
Total cost ($)	20.8		20.8		2,624.3		2.4	
Final cost C_{EP} ($ U^{-1})	0.04		0.07		2.4		0.27	

The high D_{MAX} contributed to an increase in the final LAC C_{EP}. The results showed that a low C_{CM} did not necessarily result in a low C_{EP}. This conclusion was also confirmed by [171], who demonstrated in a more detailed cost analysis that the most expensive medium using glucose was two times cheaper than the one using agricultural material (wheat bran). LAC productivity in the presence of glucose was double that in the strategy using agricultural material (Table 4-2). So, the assumption that reducing the biocatalyst production cost by using inexpensive waste material as a substrate to grow fungi and produce a low-cost enzyme hypothesis is not always true. Undeniably, the D_{MAX} plays a crucial role in the final cost. A scale-up process can contribute to a reduction in the C_{EP} by changing the process productivity. Osma et al. demonstrated that an increase of fermentation surface area by 7.5 times allows a reduction of 22% of the final cost of LAC produced by *T. pubescens* using sunflowers as a growth substrate [183]. Another approach that can be used to decrease the C_{EP} is to improve fermentation productivity by optimizing the fermentation medium and consequently decreasing the D_{MAX}. The time course of laccase in the presence of woodchips showed significant activity in the early stage of the culture compared to the D_{MAX} (Fig. 4-4). Laccase production is quite high on Day 3, which could significantly reduce the D_{MAX} and, therefore, the C_{EP}.

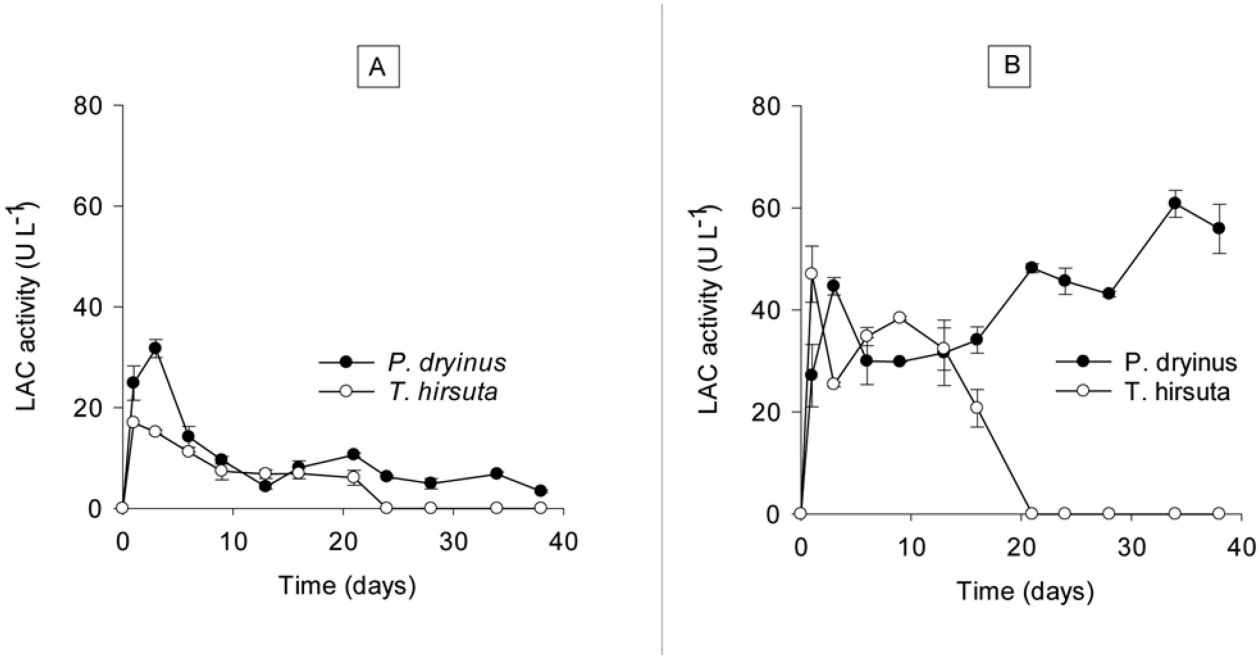

Fig. 4-4. Time course of laccase activity during mycoremediation of the biorefinery wastewater (A: Strategy 1 and B: Strategy 2). Values plotted are the means and the error bars represent the standard deviation of triplicate assays.

A comparative view of the enzyme production profile from different wastewater with the results obtained from the present study demonstrates that BRW could be valorized as a fermentation substrate for mycoremediation. From these reports, the most popular growth medium is olive mill wastewater, which is an excellent substrate for laccase production ranging from 100 to 1,000 U L^{-1} in various fungal strains [19,191]. To the best of our knowledge, this is the first study where non-sterilized BRW from treated wood biomass recycling was used as a fermentation medium for fungal growth, to achieve mycoremediation and to produce LAC. Sterilized media typically results in better enzyme production levels versus non-sterile conditions. Contrary to some published studies, the BRW demonstrates good potential for LAC production in non-sterile conditions (Table 4-4).

This study was not focused on process optimization through medium engineering and cost-effective production of LAC. Even if mycoremediation is advantageous for phenol removal in BRW, the process still requires additional research to find practical solutions to ensure the industrial-scale applicability. This study gives a global examination of the challenges in choosing the best strategy for BRW mycoremediation.

Table 4-4. A comparative view of LAC production profiles using different wastewater as a growth substrate.

Wastewater	Enrichment	Condition	Culture	Microbial source	LAC (U L^{-1})	Enzyme productivity (U L^{-1} h^{-1})	Treatment efficiency	References
Biorefinery	Glucose	Non-sterile	SmF	T. hirsuta	16.90	0.23	90 to 100% dephenolization. 25% COD	Present work
				P. dryinus	31.70	0.44		
	Woodchips			T. hirsuta	38.40	0.11		
				P. dryinus	60.80	0.07		
Olive mill	Wheat bran	Sterile	SSF	F. trogii	11,190	46.62	65% decolorization	[106]
Cheese whey					2,700	23.08	-	
Sugar					3,460	14.41	-	
Alcohol					14,180	59.08	-	
Olive mill	-	Non-sterile	SmF	P. chrysospo-rium	23	0.063	37% decolorization. 35% COD	[211]
				T. versicolor	8	0.022	84% decolorization. 83% COD	
				C. polyzona	200	0.55	73% decolorization. 71% COD	
				P. coccineus	51	0.14	81% decolorization. 81% COD	
Olive mill	Defined medium + tannic acid	Sterile	SmF	A. niger	650	21.66	70% dephenolization	[212]
Olive mill	-	Sterile	SmF	P. ostreatus	133	0.55	69-76% dephenolization	[213]
Distillery	-	Sterile	SSF	A. flavus	350	-	-	[214]
				P. ostreatus	110	-	-	

SmF: semi-solid fermentation, SSF: solid substrate fermentation, COD: dissolved organic carbon. - no data available.

4.5 Conclusion

It has been demonstrated that BRW can be used as a culture medium for both fungal growth and enzyme production, once the pH of the BRW has been adjusted to 7. All fungal strains tested here were found to produce LME and to remove both phenols and PAHs to varying degrees, with the basidiomycetes *P. dryinus* and *T. hirsuta* being the best-performing strains. The removal of these xenobiotic compounds occurred by mechanisms involving both biotransformation and biosorption of the xenobiotics, depending on the strain and the compound tested. A high removal level of phenols and PAHs was observed at detectable levels, with complete removal in some cases. A lower removal level of PCP was observed as this compound is intrinsically more recalcitrant to biodegradation. Another treatment condition has to be investigated to treat BRW to ensure the complete removal of PCP. The use of industrial wastewater in the removal strategy is significant, and the approaches employed here, involving fungal strains, offer a good starting point in the development of mycoremediation-based BRW treatment.

Authors contributions: **Arielle Farida Ariste**: Methodology, Validation, Formal analysis, Investigation, Writing-Original draft, Writing-Review & Editing, Visualization, Project administration. **Ramón Alberto Batista-García**: Methodology, Writing-Review & Editing, Visualization, Supervision. **Vinoth Kumar Vaidyanathan**: Conceptualization, Writing-Original draft, Supervision. **Nikila Raman**: Methodology, Editing. **Vasanth Kumar Vaithyanathan**: Methodology, Editing. **Jorge Luis Folch-Mallol**: Methodology, Supervision. **Stephen A. Jackson**: Methodology, Supervision. **Alan D. W. Dobson**: Methodology, Supervision, Writing-Review & Editing, Supervision. **Hubert Cabana**: Conceptualization, Validation, Resources, Writing-Review & Editing, Supervision, Project administration, Funding acquisition.

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Chapitre 5 - Évaluation d'une stratégie d'amélioration de la stabilité des lac-CLEAs

5.1 Avant-propos

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Titre français : Stratégie d'augmentation de la taille pour améliorer la stabilité des agrégats d'enzymes de réticulés : un pas en avant dans l'exploitation de la laccase pour les procédés de bioremédiation.

Contribution au document : Cet article évalue la méthode EPES comme une méthode prometteuse pour la stabilisation et le recyclage des CLEAs. Cette technologie a permis d'améliorer le potentiel d'application des CLEAs pour la bioremédiation des contaminants organiques à l'état de trace dans une eau usée municipale.

Résumé français : Malgré tous les avantages et le potentiel de la technologie des agrégats d'enzymes de réticulation (CLEA), celle-ci n'est toujours pas appliquée à l'échelle industrielle pour l'insolubilisation enzymatique à des fins de bioremédiation. Dans cette étude, la méthode EPES (Enzyme Polymer Engineered Structure) a été utilisée pour améliorer la stabilité et la réutilisation des CLEAs. Une laccase brute de *Trametes hirsuta* a été insolubilisée avec succès pour former des EPES-CLEAs. Le réseau polymère a fourni une excellente stabilité (> 90%) aux CLEAs après une incubation de 24 h dans un effluent d'eaux usées municipales brute (WW). Par centrifugation le recyclage des CLEAs et EPES-CLEAs a été évalué. Après cinq cycles d'oxydation ABTS, l'activité résiduelle des CLEAs a chuté à 17 %, tandis que les EPES-CLEA

ont montré une rétention d'activité de 67%. De plus, les EPES-CLEAs ont permis d'éliminer le cannabidiol (CBD) au même niveau que la laccase libre (free-LAC) (~37%), après 8 h de traitement dans WW. Ces travaux prouvent que la méthode EPES est une alternative prometteuse pour la stabilisation et la réutilisation des CLEAs dans des conditions environnementales.

Mots clés : Laccase, CLEAs, Stabilisation, Contaminants émergents, Efficacité d'élimination, Eaux usées.

Title: Size-increasing Strategy to Enhance Cross-linking Enzyme Aggregate Stability: A Step Forward in Laccase Exploitation in Bioremediation Processes.

Abstract: Despite all advantages and potential, Cross-linking enzyme aggregates (CLEAs) technology is still not applied at the industrial scale for enzyme insolubilization for bioremediation purposes. In this study, the enzyme polymer engineered structure (EPES) method was used to enhance CLEA stability and reuse. A crude laccase from *Trametes hirsuta* was successfully insolubilized to form EPES-CLEAs. The polymeric network provided an excellent stability (> 90%) to CLEAs after a 24-h incubation in a non-buffered municipal wastewater effluent (WW). Moreover, a centrifugation process allowed to recycle the biocatalysts. While CLEAs activity dropped to 17%, EPES-CLEAs showed a laccase activity retention of 67% after five cycles of ABTS oxidation. After 8 h treatment in WW, the EPES-CLEAs removed cannabidiol (CBD) at the same level as free-LAC (~37%). This work proves that the EPES method is a promising alternative for CLEAs stabilization and reuse in environmental conditions.

Keywords: Laccase, CLEAs, Stabilization, Emerging contaminants, Removal efficiency, Wastewater.

5.2 Introduction

The cost of the enzymes is unequivocally limiting the large-scale development of the biocatalytic process for industrial applications [177]. Therefore, extended active shelf life must be ensured with the reuse of biocatalysts for many cycles [215]. Enzyme insolubilization is one of the primary techniques for enhancing their industrial application. Physical or chemical techniques are used to generate insoluble biocatalysts by limiting the freedom of the enzyme [216]. Insolubilization allows reuse of the biocatalyst through methods such as centrifugation or filtration [158]. Insolubilization also contributes to stabilizing the enzymes by limiting the exposure of the enzymes to the denaturing environment and reducing their sensitivity to parameters such as pH, temperature or organic solvent denaturation [4].

The support-free enzyme insolubilization technique, using the cross-linking method, is a valuable technique consisting of binding the enzymes' amino-acid residues to each other, employing a cross-linker agent [137]. Enzyme cross-linking was initially developed using crystallized enzymes to produce cross-linking crystallized enzymes (CLECs). However, the demanding process of crystallization requires highly purified enzymes, which could rapidly become expensive for large-scale applications [4].

To improve the cross-linking method applicability, a strategy that does not require highly purified enzymes obtained from the precipitation of a fermentation broth was developed. Precipitation is the most common and a much less expensive technique used for enzyme isolation that can be rapidly optimized. Precipitation makes it possible to maintain the catalytic activity in a preorganized aggregate structure. Cross-linking of the aggregates forms a cross-linking enzyme aggregate (CLEAs) [28,138]. CLEA methodology does not require purified enzymes, which is a significant advantage for large-scale processes. A simple purification technique using a selective precipitation method can be combined with CLEA methodology when using crude enzyme extract. Thus, CLEAs can be prepared from the fermentation broth directly at the application site of the biocatalyst production [137].

Moreover, CLEA technology could be improved to broaden the application as it enables the combination of multiple enzymes [217]. In the past decades, fungal laccases gained attention as green biocatalysts for bioremediation purposes. Indeed, laccases only require oxygen as a cofactor to oxidize their substrates to produce water molecules [218]. This advantage makes

laccase-based biocatalysts suitable for large-scale sustainable bioremediation applications [103]. The technology was already performed for emerging contaminant removal from aqueous solutions such as spiked buffer solutions and real wastewater samples [155,219].

Overall, CLEA technology is a promising technology. However, it is still not applied in industrial scale applications because of low mechanical stability and recycling issues due to the small size of CLEA particles (in general < 100 μm) [140]. Increasing the efficiency of CLEAs involves decreasing the particle size, which in turn makes recycling difficult. Alternatives such as encapsulated CLEAs or magnetic CLEAs are techniques that have been studied as solutions to increase CLEA recovery and for reuse of nano-sized biocatalysts at the laboratory scale [34,124]. But the suitability of these alternatives for large-scale bioremediation purposes remains to be demonstrated. Therefore, increasing the size of the biocatalyst to improve recycling while maintaining catalytic efficiency should be more suitable for industrial applications. However, the encapsulation strategy suggested to increase CLEA size results in the dilution of the enzymatic activity and a subsequent decrease in the kinetic properties of the enzyme due to mass-transfer limitations [140]. Consequently, new stabilization strategies are required to make the biocatalyst effective and robust enough for real environmental conditions. The developed strategies should enable CLEAs to target large-scale applications such as emerging contaminant elimination from municipal wastewater effluents (WW).

In parallel, while research continues on the development of CLEA stabilization techniques, the ongoing detection of a new generation of contaminants adds to the complexity of the issue of emerging contaminants in the environment, such as pharmaceutical active compounds (PhACs). Recently, the Food and Drug Administration (FDA) approved a drug based on cannabidiol (CBD). CBD is the second active ingredient in cannabis, which is legalized in certain countries for medicinal or recreative practices [220]. Like PhACs, CBD molecules are known to transform in environments, and uncertainties exist regarding their fate and toxicity [221]. Thus, it is essential to evaluate the efficiency of the developed biocatalysts for the removal of emerging contaminants such as PhACs and CBD.

The main objective of this study is to evaluate a size-increasing technology for crude fungal laccase CLEA stabilization for organic contaminant removal from an aqueous solution. The method studied is an enzyme polymer engineered structure (EPES) technique. EPES is used to

form EPES-CLEAs by the formation of a polymeric network generated around the CLEAs [38]. This study addresses (i) the bioremediation potential of both CLEAs and EPES-CLEAs through physical and chemical characterization and (ii) the biocatalytic efficiency of the insolubilized biocatalysts toward acetaminophen (ACT) as a selected phenolic PhAC substrate model for laccase-based catalytic evaluation. Finally, the robustness of CLEAs and EPES-CLEAs has been assessed by (iii) testing CBD removal in a controlled environment and real municipal wastewater.

5.3 Material and methods

5.3.1 Chemicals and reagents

The high-purity LC-MS grade solvents (acetonitrile (ACN), methanol (MeOH), dichloromethane (DCM), water, formic acid (FA)), were purchased from Fisher Scientific (Ottawa, ON, Canada). All the chemicals used were of analytical grade, and were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for the CBD standard, which came from Cerilliant Corporation (Round Rock, TE, USA). A high molecular weight chitosan (CHI) powder (310-375 kDa, >75% deacetylated) was used with 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDAC) for CLEA preparation [32]. The CHI powder was initially dissolved in 0.1 M HCl at 30°C at an initial concentration of 10 g L^{-1}. The solution was filtered through a 0.45 μm and the filtrate was stored at 4°C before use for CLEA preparation. EDAC (2 M) was freshly prepared in milli-Q water and directly used for CLEA preparation. The ultra-pure water (pH 6.8, 18.2 MΩ.cm^{-1}, 25°C) produced with an ELGA Purelab System (ELGA LLC, Canada) was used as a water solvent and referred to as milli-Q water.

5.3.2 Biocatalyst preparation

Laccase was produced using the basidiomycetes strain *Trametes hirsuta (MTCC 1171*) obtained from the Microbial Type Culture Collection, India.

5.3.2.1 Crude fungal laccase production

- **Culture conditions**

The crude laccase extract was produced by the cultivation of *T. hirsuta* in a liquid medium. The fungal blended mycelium was prepared, according to [222]. Briefly, in this study, a standard medium containing 10g dextrose, 6g peptone, 3g malt extract, and 3g yeast extract in 1,000 mL of milli-Q water was sterilized (autoclaved 45 min at 121°C and 19 psi) before being used. The medium solution reached a pH of 6.5 after preparation, which is the optimum pH for *T. hirsuta (MTCC)* growth. 200 mL of this medium was dropped in 500 mL Erlenmeyer flasks, and 2.5 mL of blended mycelium (150 mg dry weight) was inoculated in each flask. The mycelium preparation was performed under aseptic conditions. The cultures were continuously stirred on an orbital shaker at 150 rpm and 27ºC.

- **Laccase partial purification from the fermentation broth**

After seven days of growth (at maximum laccase activity), the fungal biomass was discarded from the supernatant by centrifugation (4,500 rpm for 15 minutes at 4°C). The supernatant was then clarified by filtration through a 0.45 µm glass-fibre. Laccase was partially purified by precipitating the enzyme from the filtrate using ammonium sulphate at 80% saturation. The precipitated proteins were isolated by centrifugation at 4,500 rpm for 30 minutes at 4°C. The supernatant was discarded, and the precipitated protein was reconstituted in milli-Q water before overnight dialysis using a 14 kDa regenerated cellulose dialysis tubing. The purification process allowed us to obtain a laccase activity recovery of 72.0%, and a purification factor of 26.0 (data not shown). The dialysate designated as free-Laccase (free-LAC) was collected and stored at 4ºC in the dark. The free-LAC was then concentrated about ten times using 10 kDa Amicon Ultra centrifugal filters (final laccase activity of 11,000 U L^{-1}) before insolubilization. Moreover, the zymogram clearly shows two major bands of laccases on the SDS-PAGE at around 100 kDa and 40 kDa after guaiacol and ABTS oxidation (Annexe 2 : See appendix: Fig. 7).

5.3.2.2 Laccase insolubilization

The free-LAC was insolubilized and stabilized using a two-step procedure to form the EPES-CLEAs. First, the free-LAC was precipitated using ammonium sulphate and cross-linked using CHI and EDAC to form CLEAs. Secondly, APTES was used for CLEA stabilization and size-enhancer to form EPES-CLEAs.

- **CLEA preparation**

Each enzyme has a unique property regarding its amino acid residue arrangements. Therefore, the insolubilization effectiveness depends on the source of the enzyme and the insolubilization procedure [30]. A statistical analysis using experimental design (Design-Expert V11, Stat-Ease Inc, Minneapolis, MN) has been used to select the optimum parameter for the cross-linking reaction (Annexe 2 : See appendix: Fig. 8 and 9, and Tables 3 to 7). As a result, 10 mL of free-LAC was mixed with 1.7 mL of CHI solutions and 1.0 mL of EDAC. The mixture was homogenized for 10 min at 150 rpm on an orbital shaker. Then, sodium phosphate monobasic (50 mM)/sodium phosphate dibasic (50 mM) buffer pH 5 (phosphate buffer) was added to the mixture to reach a final volume of 20 mL (buffered pH ≈ 5). The reaction solution was performed at 4°C for 24 hours to complete the cross-linking reaction. The formed CLEAs were collected after centrifugation (4,500 rpm for 15 minutes). The supernatant was kept for further laccase activity measurements to determine the insolubilization yields (Table 5-1). CLEAs were then washed with 4 mL of deionized water using centrifugation at 4,500 rpm for 5 minutes. The washing step was repeated (4 times) until no laccase activity was detected in the supernatant. CLEAs were reconstituted in 10 mL of milli-Q water and stored at 4°C.

- **EPES-CLEA preparation**

CLEAs were stabilized by using APTES to form EPES-CLEAs, according to [38]. Briefly, 10 mL of CLEA solution was mixed with 10 mL of a solution containing APTES and dichloromethane (DCM) (1:10 v:v APTES:DCM). The reaction solution was vortexed for 5 minutes and then centrifuged at 4,500 rpm for 15 minutes. The supernatant was discarded, and 4 mL of milli-Q water was used to wash the biocatalyst. These washing steps were repeated (3 times) until no laccase activity was detected in the supernatant. The EPES-CLEAs obtained were stabilized at 4°C for 72 hours in a static condition before characterization or use.

5.3.3 Characterization of the biocatalysts

5.3.3.1 Laccase activity measurement

The free-LAC activity was determined spectrophotometrically by the oxidation of (3-ethylbenzthiazoline-6-sulfonic acid) 2,2-azino-bis (ABTS) to its radical cation ($ABTS^{\cdot+}$) at 420 nm (ε_{max} = 36,000 $M^{-1}.cm^{-1}$) and 20ºC according to [118]. In a microplate reader, 10 µL of

free-LAC and 90 µL of ABTS solution (1 mM) were mixed with 100 µL of pH 3 citric acid (0.1 M)/sodium phosphate monobasic (0.1 M) buffer (citrate-phosphate buffer) solution. For blank samples, 10 µL of milli-Q water was used instead of the laccase solution.

The insolubilized laccase enzymatic activity was measured through the absorbance of an aliquot. The reaction solution contained 50 µL of insolubilized laccase (CLEAs or EPES-CLEAs), dispersed in 500 µL of citrate-phosphate buffer solution (pH 3, 0.1 M) and 450 µL of ABTS solution (1 mM). The mixture was agitated at 150 rpm for 10 minutes before measurement. The samples were centrifuged for 1 min at 4,500 rpm and 20ºC to separate the supernatant and the insolubilized laccase. The supernatant was used for endpoint absorbance measurement at 420 nm at 20ºC, and the laccase activity was calculated according to [223] (Eq. 1). The collected CLEAs and EPES-CLEAs were dried overnight at 105ºC to determine the dry weight of each biocatalyst. The weight allowed us to determine the measured laccase activity as a function of the mass of the catalyst (U g^{-1} of catalysts).

$$U\ L^{-1} = \frac{(\Delta A)(V_t)(D_f)(10^6)}{(t)(\varepsilon)(d)(V_s)} \qquad \text{(Eq. 1)}$$

Where: U: enzyme activity (µmol min^{-1} mL^{-1}), ΔA: (final absorbance – initial absorbance), V_t: total volume during the reaction (mL), t: reaction time (min), ε_{max}: molar extinction coefficient (M^{-1} cm^{-1}), d: optical path (1 cm), V_s: sample volume (mL), D_f: dilution factor (included if the enzyme needs to be diluted before measurement), 10^6: correction factor (µmol to mol^{-1}).

One unit (U) of enzymatic activity is defined as the amount of enzyme that converts 1 µmole of substrate per min.

5.3.3.2 pH and temperature assay

The optimal pH and temperature were monitored through the oxidation of ABTS. The optimal pH value was determined to the range from 2 to 6 using citrate phosphate buffers (0.1 M), and phosphate buffers (0.1 M) for pH 7 to 9, and the optimal temperature was determined to range from 4 to 60°C at the optimal pH value.

The pH stability assay was conducted in environmental pHs and temperatures ranging from pH 6 to 9, and 4 to 60ºC, respectively. After 24 h of reaction on an orbital shaker at 150 rpm at the pH and temperature conditions tested, the residual laccase activity was measured.

All assays took place by using 250 µL of each catalyst incubated in 750 µL of the targeted solution.

5.3.3.3 Chemical and environmental denaturing assay

Denaturing solutions were made of 10 µM of chaotropic agents, calcium chloride ($CaCl_2$), and zinc chloride ($ZnCl_2$), and a chelating agent, ethylenediaminetetraacetic acid (EDTA). A water-miscible organic solvent made of 25% v/v methanol (MeOH) was also tested. The target application of the biocatalysts was to be applied for PhAC removal from WW. So, their stability was also tested in a 100% WW from a local municipal wastewater treatment plant (Magog, Québec, Canada) (Annexe 2 : See appendix: Table 8). 250 µL of each biocatalyst was incubated in 750 µL of the denaturing solution. After 24-h of reaction on an orbital shaker at 150 rpm at optimal pH and 20ºC, the laccase activity was measured.

Kinetic parameters of free-LAC, CLEAs and EPES-CLEAs were determined in pH 3 citrate phosphate buffer solution (0.1 M) at 20ºC by increasing ABTS concentration from a 0.05 mM to 2 mM 8-point calibration dissolved. Michaelis-Menten constant Km and Kcat were obtained by curve fitting of the Michaelis-Menten equation from experimental data using Microsoft Excel (2016) (Annexe 2: See appendix: Eq. 2, 3 and 4).

5.3.3.4 Physical characterization

The size distributions of CLEAs and EPES-CLEAs were determined using a Malvern Mastersizer 2000 laser diffraction analyzer (Malvern Instruments, Malvern, UK). Particle size was measured using the following optimum conditions: a particle refractive index of 1.5, a dispersant refractive index of 1.3 and a particle absorption index of 0.1.

CLEAs and EPES-CLEAs were subjected to an electron dispersive spectroscopy (EDS) analysis. An Hitachi S4700 analyzer (Hitachi Ltd., Tokyo, Japan) was used at 20 kV. A few microliters (~20 µL) of suspended CLEAs and EPES-CLEAs were dried at room temperature for the elemental identification analysis.

5.3.3.5 Reusability assessments

The reusability of CLEAs and EPES-CLEAs was assessed during 5 successive cycles of ABTS oxidation (as described in section 5.2.3.1). One cycle was considered as a 10-min reaction time of the ABTS oxidation. After each cycle, the CLEAs and EPES-CLEAs were thoroughly washed (5 times during 1 min) with milli-Q water and centrifuged to remove the remaining ABTS from the biocatalysts altogether. Triplicate assays were performed, and the relative laccase activity was determined by the ratio of measured laccase activity after each cycle and that of the initial one.

5.3.4 Organic contaminant removal

The efficiency of the biocatalysts to eliminate ACT as a model phenolic compound and CBD as an emerging contaminant of interest has been assessed in a controlled environment using phosphate buffer solution (pH 7, 0.1 M). The robustness of the biocatalysts was also assessed through CBD removal by using a WW solution.

5.3.4.1 Evaluation of removal efficiency with a model phenolic compound

The experiment was performed in 1.5 mL Eppendorf containing 1 mL of pH 7 phosphate buffer solution (0.1 M). An initial concentration of 0.662 μM of ACT was spiked in each Eppendorf. 0.1 U mL^{-1} of each biocatalyst was used for the catalytic experiment. The samples were placed in the dark at 20ºC on an orbital shaker at 135 rpm, at a regular time interval (0, 1, 2, 4, 6, 8, 24 and 48 h), and one triplicate of each sample was used to determine ACT removal.

5.3.4.2 Cannabidiol elimination and biocatalyst robustness assay

The experiments were carried out to evaluate the efficiency of laccase to eliminate CBD from aqueous solutions using two experimental setups. The first set of experiments was carried out in a 1.5 mL Eppendorf using a simple buffered solution (pH 7 phosphate buffer (0.1 M)). The second set of experiments took place in WW to evaluate the efficiency of the biocatalysts to eliminate CBD in a real WW solution. Each sample (final volume of 1 mL) contained 0.1 U mL^{-1} of laccase and a final CBD concentration of 0.318 μM for both sets of the experiments. For all conditions studied, the effect of ACT as a model phenolic organic contaminant on CBD removal

was evaluated by the addition of ACT to the reaction mixture at two concentrations (0.1 and 1 mM). The experiments were conducted for 8 h at 20ºC in the dark and under agitation on a rotary shaker (135 rpm). One triplicate of each condition was sacrificed for analysis at the end of the reaction time.

All experiments were performed using control samples made of active and inactivated (heat and sodium azide deactivation) biocatalysts, which allowed for sorption, removal and compound stability to be considered during the experiment.

5.3.5 Organic contaminants analysis

Briefly, ACT and CBD were extracted using a solid-phase extraction method and filtered through a 0.22 µm PTFE syringe filter. They were then analyzed with liquid chromatography coupled to a tandem mass spectrometer using a positive/negative electrospray ionization (ESI) source in the Multiple-Reaction-Monitoring mode (MRM) (Annexe 2 : See appendix: Methods 1 and 2). The control samples were used to correct the removal observed in order to report the effective removal of each biocatalyst. The reaction mixture containing the contaminants and inactivated biocatalysts served as controls. Individual abiotic controls containing only the studied contaminants and the studied biocatalysts were also used. Results are expressed as the means of triplicate values, and the error bars represent the standard deviation.

5.4 Results and discussion

5.4.1 Laccase insolubilization

Laccase insolubilization yield, efficiency, and activity recovery were calculated (details of the calculation are provided in (Annexe 2 : See appendix: Eq. 5, 6 and 7). The insolubilization yield was determined to be approximately 70% (69.80% for CLEAs and 68,40% for EPES-CLEAs) (Table 5-1). Nevertheless, a significant difference was observed in the measured activity of insolubilization efficiency and activity recovery (Table 5-1). The efficiency was 61.70% and 3.90% for CLEAs and EPES-CLEAs, respectively. The activity recovery reached 43.10% and 2.60% for CLEAs and EPES-CLEAs, respectively (Table 1). Thus, having a 100% yield did not directly involve either high activity recovery or efficiency [130]. An activity recovery of 1% using a commercial laccase of *T. versicolor* was already reported [38]. Arsenault and co-workers

observed a reduction of laccase activity when preparing CLEAs from a CHI concentration higher than 1 g L^{-1} [32]. The studied technology leads to the preparation of CLEAs and EPES-CLEAs with a specific activity (unit per gram of biocatalyst) of 417.30 U g^{-1} and 28.10 U g^{-1}, respectively (Table 5-1).

The decrease in the measured enzymatic activity can be explained by several factors such as enzyme deactivation during the insolubilization procedure, changes in the three-dimensional structure of the enzyme and/or mass transfer limitations. All these factors can prevent the substrate from reaching the active sites of the enzyme after insolubilization [138]. CLEAs have a highly porous structure, which results in diffusional limitations. The particle size can affect the rate of diffusion. Therefore, the bigger the particle size, the higher the diffusion limitations. The EPES method is proposed here as a size-increasing strategy to stabilize CLEAs. The enzymatic activity of insolubilized enzymes showing low activity such as EPES-CLEAs should be discussed with caution. Indeed, the mass transfer limitations may hamper the real potential of the biocatalyst.

Tableau 5-1. CLEA and EPES-CLEA insolubilization performances.

Parameters	CLEAs	EPES-CLEAs
Yield (%)	69.80 ±1.90	68.40 ±1.80
Efficiency (%)	61.70 ±1.10	3.90 ±0.10
Activity recovery (%)	43.10 ±1.10	2.60 ±0.10
Specific activity (U g^{-1})	417.30 ±11.00	28.10 ±0.50

Nowadays, it is still difficult to compare the published CLEA insolubilization yields to each other because of a lack of comparable data. Uniformization of CLEA data based on the target application should then allow a better characterization and comparison of the CLEAs and consequently contribute to a step towards their industrial applications.

5.4.2 Biocatalyst characterization

5.4.2.1 Effect of pH on biocatalyst activity

The optimum pH value for all biocatalysts was found to be pH 3 (Annexe 2 : See appendix: Fig. 10). Beyond the optimum pH value, laccase activity decreases until complete inhibition above

pH7 (Annexe 2 : See appendix: Fig. 10). These results are in accordance with the literature where optimal pH values for most free fungal laccases are observed in the acidic region from pH 3 to pH 6 [155]. This inhibition is known to be caused by an interruption of the internal electron transfer from T1 to T2/T3 copper sites of laccase through the binding of hydroxide anions to the T2/T3 copper [224]. A few previous works have shown that CLEA preparation could result in a shift of the optimum pH towards the alkaline region [39,225]. Such a shift also was observed after laccase insolubilization using chitosan [38]. The ionic interaction between enzymes and charged insolubilization agents leads to a microenvironment that could change the protonation pattern of the enzyme and consequently alter the enzyme structure and activity [135]. From this result, pH 3 was used in the study as an optimum value for laccase activity measurement.

Insolubilization contributes to enhancing laccase stability towards the environmental pH range (pH 6 to pH 9) after a 24-h incubation. As shown in Fig. 5-1, the free-LAC biocatalyst decreased under 60% of its enzymatic activity from pH 6 to pH 8 and decreased around 30% at pH 9. It has been demonstrated that at pH values higher than 6.5, fungal laccases are poorly active or inactive [224]. Thus, free-LAC is not directly suitable for environmental conditions.

Interestingly, CLEAs and EPES-CLEAs maintained over 90% of their initial activity across the tested pH (Fig. 5-1). These data showed that CLEA and EPES-CLEA stability toward pH was the same. The results demonstrate that the covalent binding between the laccase and CHI provided a stable conformation to pH variation. The EPES method didn't affect the high pH stability of CLEA. The buffering impact provided by the polymeric network surrounding the enzyme could explain the higher stability of the insolubilized laccase [226].

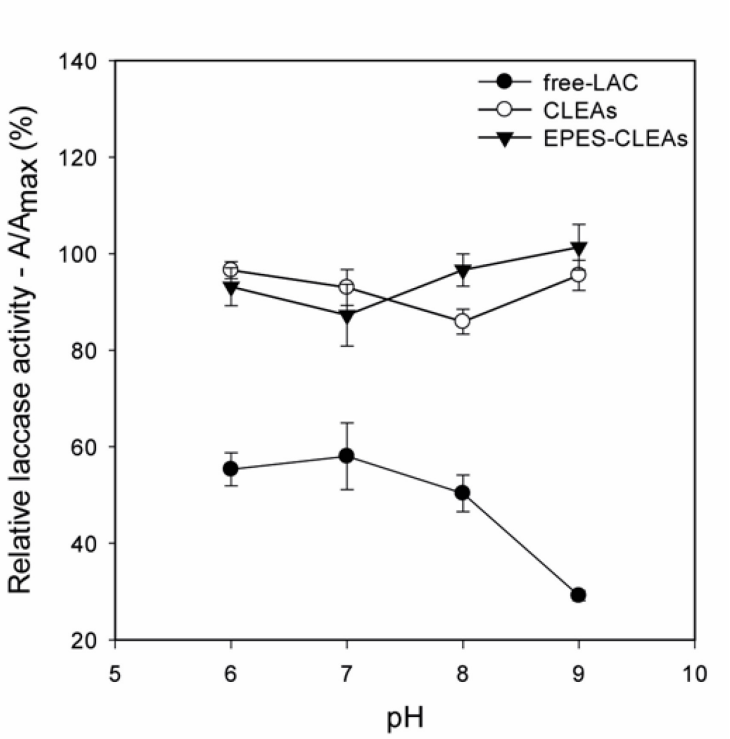

Fig. 5-1. Laccase activity profile as a function of pH after a 24-h incubation at 20ºC. Each value plotted represents the means of the triplicate sample with the error bars representing the standard deviation.

The regulation on municipal wastewater treatment plants in Quebec sets the standard for the pH value of wastewaters between 6 and 9.5. Therefore, the application of CLEAs and EPES-CLEAs for environmental pH conditions such as the one encountered for WW is highly recommended.

5.4.2.2 Effect of temperature on biocatalyst activity

The efficiency of the biocatalysts at different temperatures was evaluated to determine the optimum values. From 20ºC to 60ºC, CLEA activity remains higher than 90%, while the relative activities of free-LAC and EPES-CLEA reach a maximum at 40ºC before decreasing (Annexe 2 : See appendix: Fig. 11).

After 24 h of incubation at a different temperature, all biocatalyst activities decreased with the increase in temperature, as showed in Fig. 5-2. Compared to free-LAC, insolubilization contributes to stabilizing laccase with higher residual activity over a wide range of temperatures. The residual activity for free-LAC was maintained around 60% until 40ºC (Fig. 5-2). CLEA

and EPES-CLEA activities were higher at 4ºC to 20 ºC with 101% and 108%, respectively, before a decrease from 20ºC with the increase in temperature (Fig. 5-2). Free-LAC and CLEA activities dropped from 46% and 75% at 40ºC to reach 14% and 20% at 60ºC, respectively, while EPES-CLEA activity remained higher, with 79% of its initial activity (Fig. 5-2). The covalent bond formation helps to preserve the insolubilized enzyme for conformational changes through the rigidification of its structure [227].

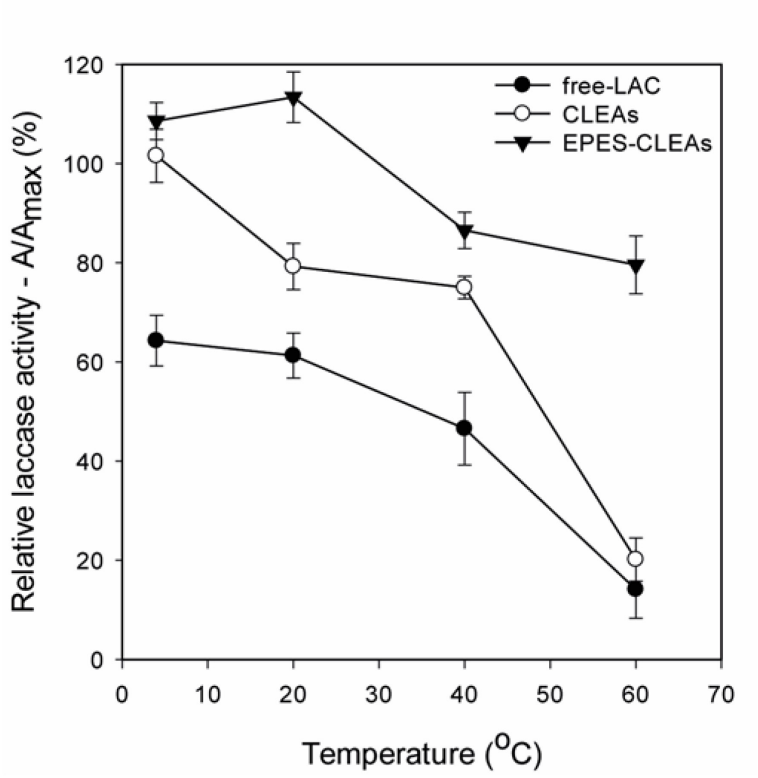

Fig. 5-2. Laccase activity profile as a function of temperature after 24 h of incubation in sodium phosphate monobasic/sodium phosphate dibasic 0.1 M buffer at pH 7. Each value plotted represents the means of the triplicate sample with the error bars representing the standard deviation.

The EPES method provided high stability to CLEAs over 40ºC. The insolubilization process is prompt to increase the overall stability of the CLEAs through the protection provided by the polymeric cage against the temperature changes in the environment surrounding the biocatalyst [228].

5.4.2.3 Biocatalyst stability against chemical and environmental denaturants

Fig. 5-3 shows the stability of the biocatalysts after a 24-h exposure to selected chaotropic salts ($CaCl_2$, $ZnCl_2$), a chelator (EDTA), a water-miscible organic solvent (MeOH) and a WW. The insolubilization helped maintain laccase activity regarding the studied denaturants (Fig. 5-3).

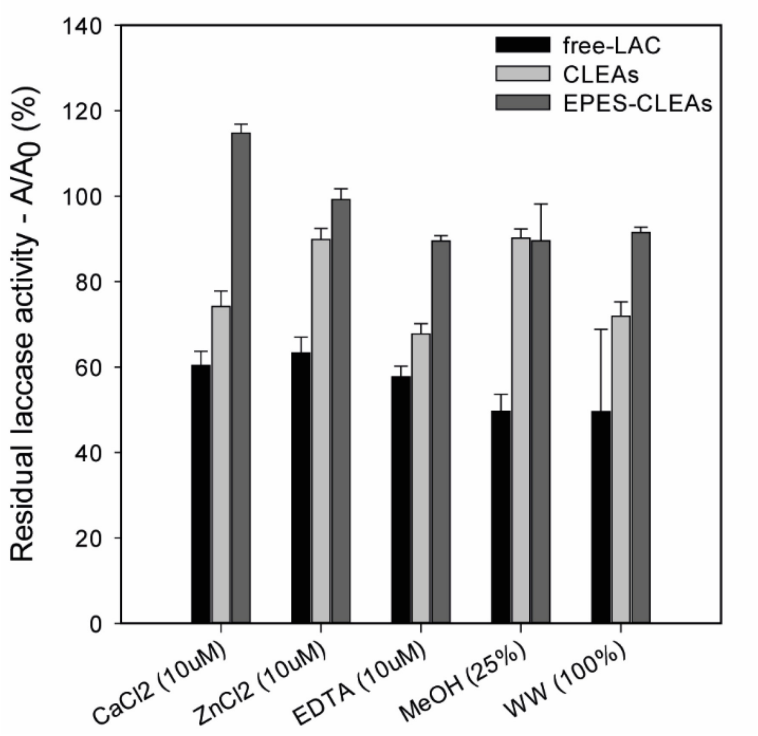

Fig. 5-3. Residual laccase activity after 24 h of incubation at 20ºC for free-LAC, CLEAs and EPES-CLEAs in the presence of denaturing agents in sodium phosphate monobasic/sodium phosphate dibasic 0.1 M buffer at pH 7. Each value represents the means of the triplicate sample with the error bars representing the standard deviation.

The residual activity of free-LAC and CLEAs was significantly impacted by the presence of both chaotropic salts. The free-LAC showed the lowest residual activity with the same activity, around 60% in the presence of $CaCl_2$ and $ZnCl_2$. CLEA activity appeared to be more sensitive to $CaCl_2$ (74%) than to $ZnCl_2$ (89%). In the presence of both salts, EPES-CLEAs were able to maintain their initial laccase activity (Fig. 5-3). These results could be explained by changes in the ionic strength of the solution that could inhibit laccase activity [229].

When incubated in the presence of EDTA, free-LAC, CLEAs, and EPES-CLEAs showed 57%, 67% and 89% residual activity, respectively (Fig. 5-3). In the presence of MeOH, free-LAC lost

around half of its activity, while CLEAs and EPES-CLEAs maintained 90% of their initial activity (Fig. 5-3). The enhancement of laccase stability towards these chemicals' denaturants could be attributed to the polymeric cage made of CHI and APTES that contribute to reducing the transfer of denaturing agents from the solution to the enzyme and thus protect the active site of the enzyme.

Interestingly, the results obtained with WW showed that environmental matrices significantly impact the efficiency of free-LAC and CLEAs by decreasing the residual activity by about 50% and 25%, respectively. EPES-CLEAs exhibited the highest residual activity, with only a 5% loss (Fig 5-3). Again, the results highlight that the EPES method contributes to increasing CLEA stability toward environmental conditions.

The activity and the stability of the biocatalysts involved in the enzymatic bioprocess is one of the crucial elements that must be validated for enzymatic bioremediation purposes. These results support that insolubilization prevents the inhibition of free-LAC from denaturing agents in the tested conditions, and that the EPES method improved CLEA stability, making EPES-CLEAs a biocatalyst of choice for conditions typically met in the environment.

5.4.2.4 Kinetics parameters

The Michaelis-Menten constant values for free-LAC, CLEAs and EPES-CLEAs are provided in Table 5-2. The results showed that the Km value increased after insolubilization and stabilization. The Km value was 17.0 μM for free-LAC, 103.9 μM for CLEAs and 107.4 μM for EPES-CLEAs (Table 5-2). The Km values for CLEAs and EPES-CLEAs did not have a significant difference ($p \leq 0.05$), indicating that the EPES method did not induce additional significant diffusion limitations to the CLEAs. Compared to free-LAC, the increase in Km values of CLEAs and EPES-CLEAs indicates a lower substrate (ABTS) affinity (up to 6-fold lower) (Table 5-2). The measured turnover number (Kcat) of free-LAC (1.014 μmole min^{-1} U^{-1}) is higher than that of CLEAs (0.901 μmole min^{-1} U^{-1}) as well as of EPES-CLEAs (0.712 μmole min^{-1} U^{-1}). This observation indicates that the insolubilization process decreases the active sites of the enzyme by 10% for CLEAs and 30% for EPES-CLEAs compared to free-LAC (Table 5-2). The catalytic efficiency (Kcat/Km) showed that CLEA stabilization leads to

a decrease in the kinetics parameters. Indeed, 6.7-fold and 8.6-fold lower efficiencies than free-LAC were observed after insolubilization (Table 5-2).

Tableau 5-2. Michaelis-Menten kinetic constants of free-LAC, CLEAs and EPES-CLEAs. Value represents the means of triplicate standard ± deviation obtained from ABTS oxidation at pH 3 and 20°C.

	Parameters	free-LAC	CLEAs	EPES-CLEAs
Kinetics parameters	Km (μM)	17.0 ±1.0	103.9 ±1.0	107.4 ±6.0
	Kcat (μmole min^{-1} U^{-1})	1.014 ±0.018	0.901 ±0.012	0.712 ±0.066
	Kcat/Km (L U^{-1} min^{-1})	0.060 ±0.018	0.009 ±0.012	0.007 ±0.001
	R^2	0.90	0.96	0.98

The results revealed that the EPES method decreased the catalytic properties of CLEAs by 20% but provided a stable biocatalyst with efficient catalytic capabilities. Steric hindrances or the substrate diffusion limitations are factors readily contributing to a decrease in the accessibility of the enzyme active site after enzyme insolubilization [230]. The particle size is also known to influence the diffusion rate of the substrate through the biocatalyst structure [138].

5.4.2.5 EDS characterization and size distribution of CLEAs and EPES-CLEAs

The result of the elemental EDS analysis confirms the successful silanization of CLEAs with APTES to form EPES-CLEAs. The results presented show the presence of silica on the surface of the EPES-CLEAs. In contrast, no elemental silica was detected on the surface of the CLEAs (Annexe 2 : See appendix: Fig. 12).

As presented in Fig. 5-4, the particle size of the formed CLEAs, and EPES-CLEAs varied from 1 μm to 1000 μm. The average size was around 60 μm for CLEAs and 130 μm for EPES-CLEAs confirming the increasing particle size of the EPES method (Fig. 5-4). The CLEA particle size is known to significantly influence the activity and effectiveness of the biocatalyst. Unfortunately, the size distribution of insolubilized laccase is not systematically evaluated when preparing CLEAs. Such information is still missing in the literature and remains to be addressed in order to perform a normalized comparison between CLEA efficiencies. These facts could significantly impact the step forward to CLEA industrialization. The reported studies show that

the insolubilized laccase particle size using different CLEA preparation strategies, varied between 0.1 to 200 μm [140]. In addition, the narrow distribution of EPES-CLEAs suggested that the EPES method results in the formation of a more uniform particle size distribution compared to CLEAs (Fig. 5-4). A uniform and large biocatalyst should facilitate the biocatalyst's recyclability and help control the mass transfer limitation, which is beneficial for industrial applications [28,231].

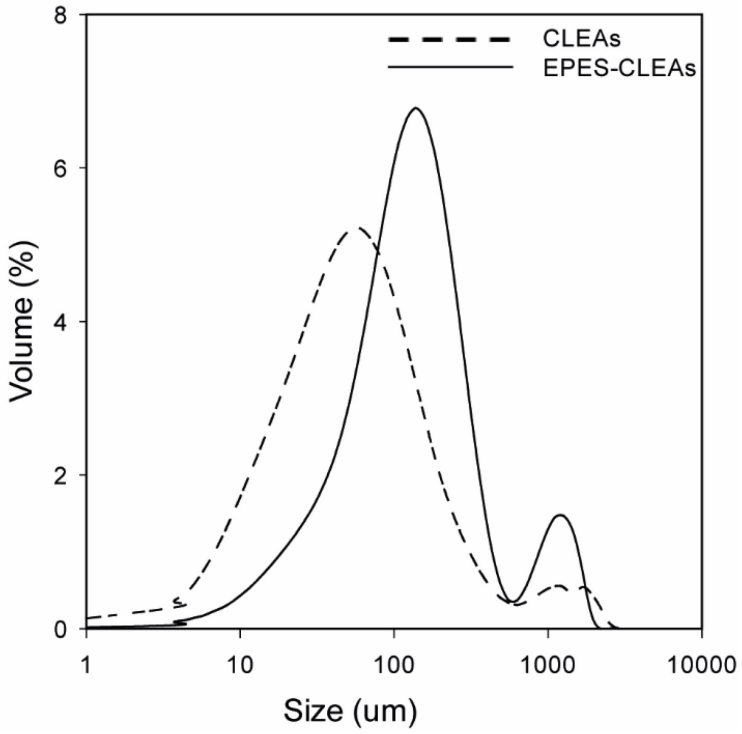

Fig. 5-4. Size distributions of CLEAs and EPES-CLEAs.

Overall, the structure (size and shape) of the insolubilized enzyme is an essential characteristic of a direct effect on the biocatalyst catalytic properties and recyclability. These results show that particle size plays a major role in the overall parameters. However, further research is needed to study the effect of the particle shape of the insolubilized enzyme on their physicochemical properties.

5.4.2.6 Reusability

The industrial reuse of oxidative enzymes for many reaction cycles should be the primary goal of enzyme insolubilization. The recycling potential of CLEAs and EPES-CLEAs was investigated using a common centrifugation process as a recycling technique, as described in

Section 2.3.5. The results showed that EPES-CLEAs are more stable in the recovery process than CLEAs (Fig. 5-5). After 5 oxidation cycles using ABTS as substrate, the EPES-CLEAs maintained 67% of their initial laccase activity while CLEA activity dropped to 17% (Fig. 5-5). Moreover, from the second cycle, the residual activity drastically diminished to 26% for CLEAs, while only a slight decrease was observed for EPES-CLEAs with 81%.

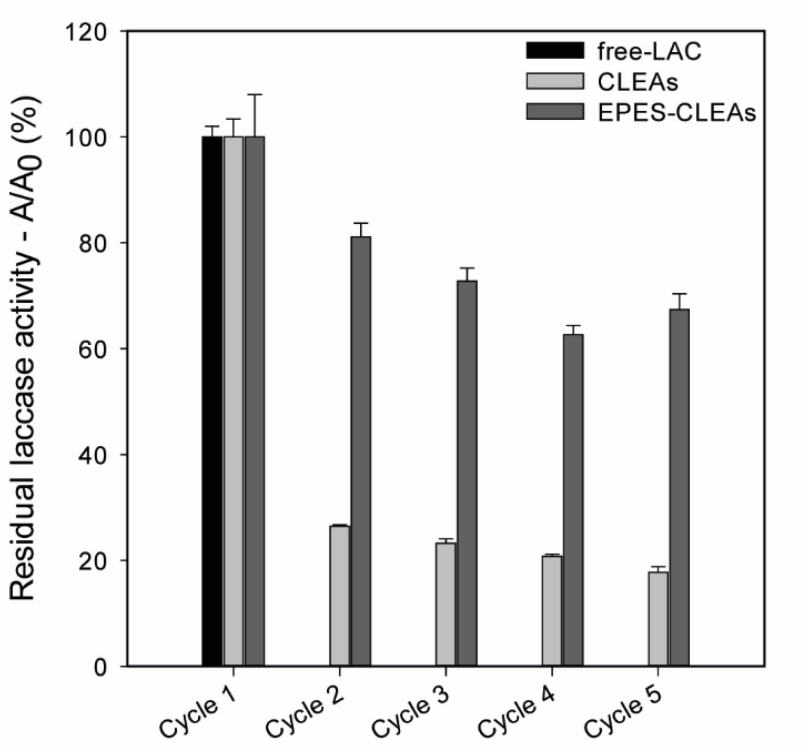

Fig. 5-5. Residual activity for CLEAs and EPES-CLEAs during five recycles with ABTS as the substrate in sodium phosphate monobasic/sodium phosphate dibasic 0.1 M buffer at pH 7 at 20ºC. The values represent the means of the triplicate sample with the error bars representing the standard deviation.

In practice, the recovery of the biocatalysts is facilitated by a larger particle size [28]. The additional polymer layer made of APTES contributes to an increase in the size of CLEA particles, as presented in Fig. 5-4. However, as previously discussed (Section 5.3.2.4), an increase in CLEA particle size contributes to reducing the catalytic efficiency of the biocatalyst through mass transfer and/or access to the active site limitations. Consequently, to the best of our knowledge, a compromise must be made between the catalytic properties of the biocatalyst and its recovery.

Centrifugation is a standard method used in the industry for biocatalyst recovery during preparation or during the recycling process [232]. However, the centrifugal force can also cause CLEA aggregation. It could lead to the formation of clusters, which can contribute to aggregating the CLEA particles and subsequently decrease the biocatalyst activity and accentuate mass-transfer limitations [140]. The wider distribution of CLEA particle size could illustrate the aggregation phenomena toward the narrow particle size obtained with EPES-CLEAs (Fig. 5-4). Our results suggest that EPES-CLEAs are more suitable when reusability is an issue and demonstrate that the EPES method contributes to enhancing CLEA stability and application.

5.4.3 Batch elimination of organic contaminants from aqueous solutions

Laccase efficiency for contaminant removal from water depends on a multifactorial condition, such as, among others, the chemical structure of the target contaminant and the presence of other compounds. ACT and CBD have a hydroxyl (OH) functional group in their chemical structure, which could make them suitable substrates for laccases [83,221]. The efficiency of the studied biocatalysts to eliminate both contaminants have been tested in the following sections.

5.4.3.1 Model phenolic compound removal from a buffered solution

ACT has been selected as a model compound to assess the effectiveness of the presented biocatalysts for phenolic PhAC elimination in water. No significant pH variation was observed during the reaction time. All the studied biocatalyst activities were maintained to around 100% of the initial activity (Fig. 5-6a). Regarding ACT removal, after 8 h of reaction, the free-LAC achieved 100% elimination, while both CLEAs and EPES-CLEAs reached 40% and 25%, respectively, after 48 h of treatment (Fig. 5-6b). These results are consistent with the literature where a significant decrease in the biocatalyst's efficiency was observed for the insolubilized enzyme. Recently, 80% and 34% removal using ACT as a subtract for free-LAC and CLEAs after 6 h of treatment were reported [124].

The elimination rate of acetaminophen was calculated for all the biocatalysts studied. The results confirmed that free-LAC was 100 times more efficient in acetaminophen elimination with 1.45×10^{-03} μmole h^{-1} U^{-1}, than CLEAs and EPES-CLEAs which showed 4.9×10^{-05} μmole h^{-1} U^{-1} and 2.3×10^{-05} μmole h^{-1} U^{-1} elimination, respectively. A two-fold increase in the average

particle size of CLEAs leads to a two-fold decrease in ACT removal efficiency. However, an experiment with multiple particle sizes must be performed in order to support such an assumption, as the insolubilized biocatalysts showed a wide range of sizes (from 1 to 1000 μm) as showed in Fig. 5-4.

5.4.3.2 Cannabidiol elimination and evaluation of the presence of acetaminophen

- **Cannabidiol elimination from a buffer solution**

In a simple controlled environment of sodium phosphate monobasic/sodium phosphate dibasic 0.1 M, pH 7 buffer solution (PBS), free-LAC was more effective in CBD elimination compared to CLEAs and EPES-CLEAs (Fig. 5-6c). After 8 h of treatment, the free-LAC was able to remove 35% of CBD, while the CLEAs removed 25% of CBD and the EPES-CLEAs were only able to remove 4% of CBD (Fig. 5-6c). These results confirmed that insolubilization readily influences biocatalyst effectiveness by decreasing the catalytic efficiency of the insolubilized biocatalysts as previously supposed (Table 5-2). After 8 h of reaction, CLEAs were around 5 times more efficient than EPES-CLEAs regarding CBD removal in PBS (Fig. 5-6c). This result can be explained by the combined effect of all phenomena previously discussed, such as mass-transfer limitations or rigidification of the enzyme structure after stabilization at the tested conditions.

It has been demonstrated that in the presence of a complex environment, containing easily oxidizable phenolic compounds such as WW, the removal of CBD is enhanced. The transformation of the easily oxidable laccase substrate ACT by laccase, formed radicals and oligomers [125]. The radicals derived from this compound are known to act as electron shuttles, enabling laccases to indirectly eliminate weakly oxidized compounds by possible cross-coupling phenomena [126]. Therefore, the effect of ACT has been tested to assess its impact on CBD elimination in PBS but also in WW.

Fig. 6c the elimination of CBD in PBS after the 8 h of reaction. The results showed significant CBD elimination for all the tested biocatalysts. In the presence of ACT at 0.1 mM, EPES-CLEAs were the most efficient in eliminating CBD with 65% elimination. The free-LAC and CLEAs showed 52% and 51% CBD elimination, respectively (Fig. 5-6c). At a higher concentration of ACT (1 mM), the elimination of CBD by EPES-CLEAs remains approximately the same with 63% CBD removal. At the same time, free-LAC and CLEA efficiencies were

84% and 70%, respectively (Fig. 5-6c). This result could be explained by the cross-coupling mechanism of ACT toward the recalcitrant PhAC carbamazepine reported by [127].

- **Cannabidiol elimination from municipal wastewater**

The effect of a real WW matrix in the removal of CBD was evaluated in a non-buffered WW (Fig. 5-6d). The results showed that all the biocatalysts were able to eliminate CBD at a significant level (> 38%). Free-LAC and EPES-CLEAs were able to eliminate CBD at approximately the same level, with 38% and 37%, respectively, while, CLEAs showed the highest CBD elimination with 50% removal (Fig. 5-6d). As previously discussed, WW can provide an environment that could inhibit or enhance enzymatic activity (Section 5.3.2.3). These results demonstrated that WW has a positive effect on CBD removal. Indeed, the insolubilized laccase was more effective in eliminating CBD in the WW (Fig. 5-6d) than in the PBS (Fig. 5-6d). It has been demonstrated that some compounds, such as natural organic matter and humic substances, and other small organic contaminants present in WW, can act as mediators, and contribute to enhancing laccase-based oxidation of some emerging recalcitrant contaminants [120,233].

Fig. 5-6d shows the elimination of CBD in the WW in the presence of ACT. The free-LAC was the most efficient at removing CBD with 68% and 92% elimination in the presence of 0.1 mM and 1 mM ACT concentration, respectively (Fig. 5-6d). CLEA and EPES-CLEA efficiencies were not radically influenced by the concentration of ACT. Indeed, CLEAs eliminate 42% and 44%, while EPES-CLEAs eliminate 36% and 30%, in the presence of 0.1 mM and 1 mM ACT, respectively (Fig. 5-6d).

The comparison of both Fig. 5-6c, and Fig. 5-6d suggests that the efficiency of free-LAC was influenced by the presence and concentration of ACT in WW. Regarding CLEAs and EPES-CLEAs, the presence of ACT did not enhance their capability to eliminate CBD in WW. CLEA efficiency slightly decreases by about 10% in the presence of ACT compared to the experiment in WW without ACT. EPES-CLEA efficiency toward CBD remained the same at around 38% in all studied conditions in WW. Overall, these interesting results suggested that the competitive substrates present in WW could be limiting the mediator effect observed by the presence of ACT on CBD removal in the WW (Fig. 5-6).

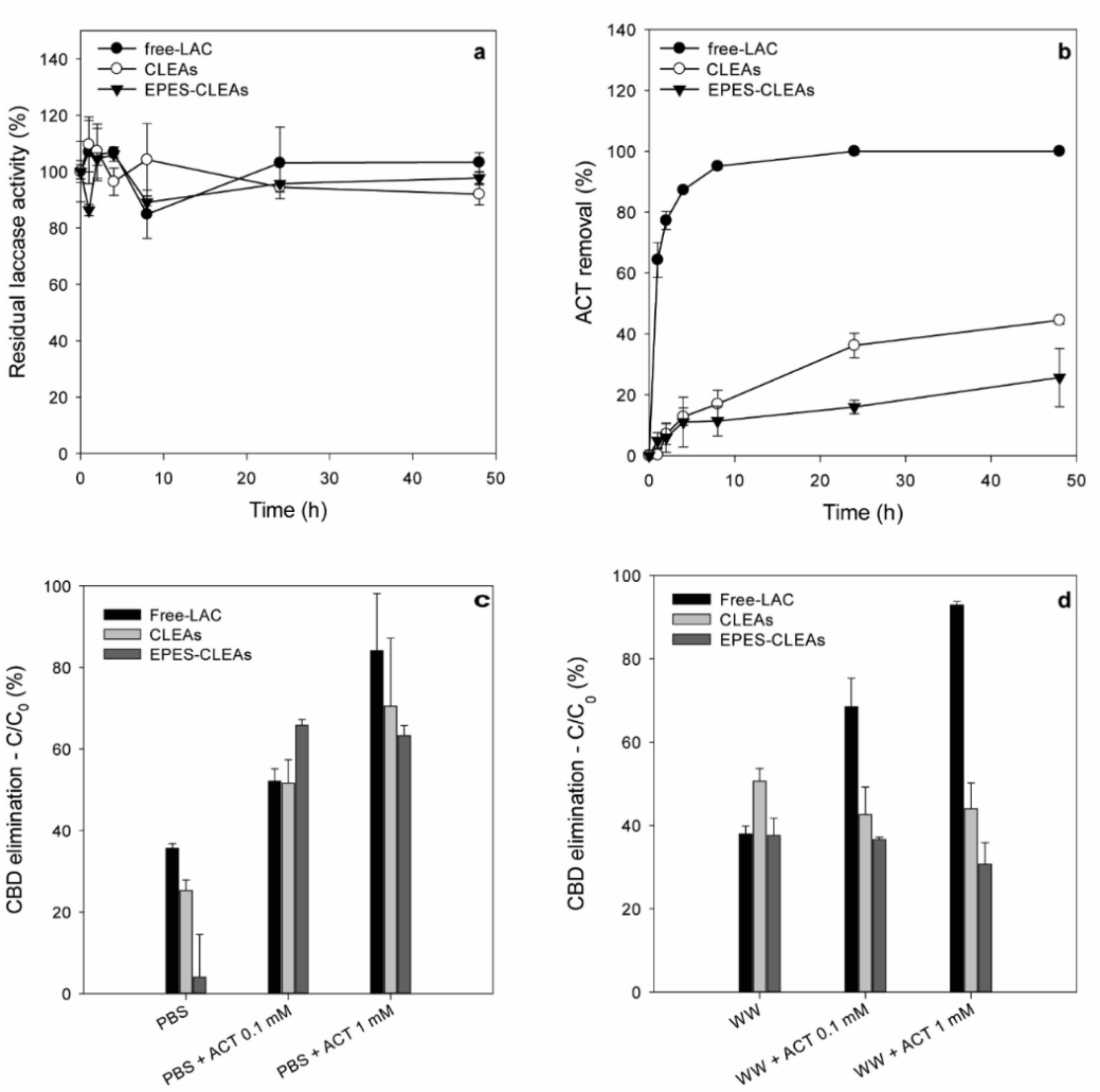

Fig. 5-6. Time course residual laccase activity (Fig. 5-6a) and acetaminophen removal (Fig. 5-6b) from sodium phosphate monobasic/sodium phosphate dibasic 0.1 M buffer at pH 7 and 20ºC by free-LAC, CLEAs and EPES-CLEAs. Cannabidiol (CBD) elimination in sodium phosphate monobasic/sodium phosphate dibasic 0.1 M buffer at pH 7 (PBS) and evaluation of the presence of acetaminophen (ACT) on CBD elimination from PBS (Fig. 5-6c). CBD elimination in non-buffered municipal wastewater effluent (WW) and evaluation of the presence of acetaminophen (ACT) on CBD elimination from WW (Fig. 5-6d). All free-LAC, CLEAs and EPES-CLEAs were used for 8 h of treatment at 20ºC for CBD removal. Each value represents the means of the triplicate sample with the error bars representing the standard deviation.

To the best of our knowledge, this is the first time that results from the use of an isolated *T. hirsuta* laccase for CBD elimination in aqueous solutions have been presented. These results also highlight the beneficial environment offered by WW and open the gate to this more precise research area regarding the industrial applicability of CLEAs for bioremediation of emerging contaminants.

5.5 Conclusion

In this study, a crude isolated laccase from *T. hirsuta* was successfully insolubilized using a size-increasing strategy of CLEAs made with CHI using a polymeric network made of APTES to form the so-called EPES-CLEAs. The method studied resulted in the formation of EPES-CLEAs with an average particle size twice that of CLEAs. The characterization of free-LAC, CLEAs and EPES-CLEAs indicated that the EPES method significantly enhanced the enzyme stability regarding environmental ranges of pH and temperature, and denaturing conditions with higher stability in real non-buffered WW. The EPES method also helped to enhance CLEA recyclability. The higher recyclability of EPES-CLEAs also contributes to emphasizing its potential for industrial applications.

This work also demonstrated, the application of EPES-CLEAs in PhAC elimination in aqueous solutions. The results showed the capacity of EPES-CLEAs to remove ACT and CBD from PBS (pH7) and non-buffered real WW. The present technology indicates that EPES-CLEAs is a promising technology for environmental applications of CLEAs. However, as a size-increasing approach, the EPES method increases the mass-transfer limitations of CLEAs. Therefore, further research is needed to optimize the catalytic performances of EPES-CLEAs.

Author contributions: All authors contributed to the study conception and methodology. Investigation, formal analysis and writing – original draft was performed by **AFA**. **LH** contributed to the methodology and data presentation. **SS** contributed to the investigation. The supervision, project administration and funding acquisition were ensured by **HC. All** authors read and approved the final manuscript.

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Chapitre 6 - Estimation des coûts de production des EPS-CLEAs

Ce chapitre a pour objectif de fournir une estimation des coûts de production des biocatalyseurs étudiés au chapitre 5 (free-LAC, CLEAs et EPES-CLEAs) à grande échelle. Cette amorce préliminaire a pour finalité d'identifier les facteurs clés qui pourraient sur le plan économique limiter l'application à grande échelle de ces biocatalyseurs.

6.1 Méthodologie

L'estimation des coûts de production du biocatalyseur a été réalisée en considérant chaque étape du processus (fermentation, purification et insolubilisation) (Figure 6-1). Le principe du procédé repose sur la production d'un extrait brut de laccase (free-LAC) et de son insolubilisation sous forme de CLEAs et EPES-CLEAs. Les valeurs retenues sont celles décrites dans le chapitre 5.

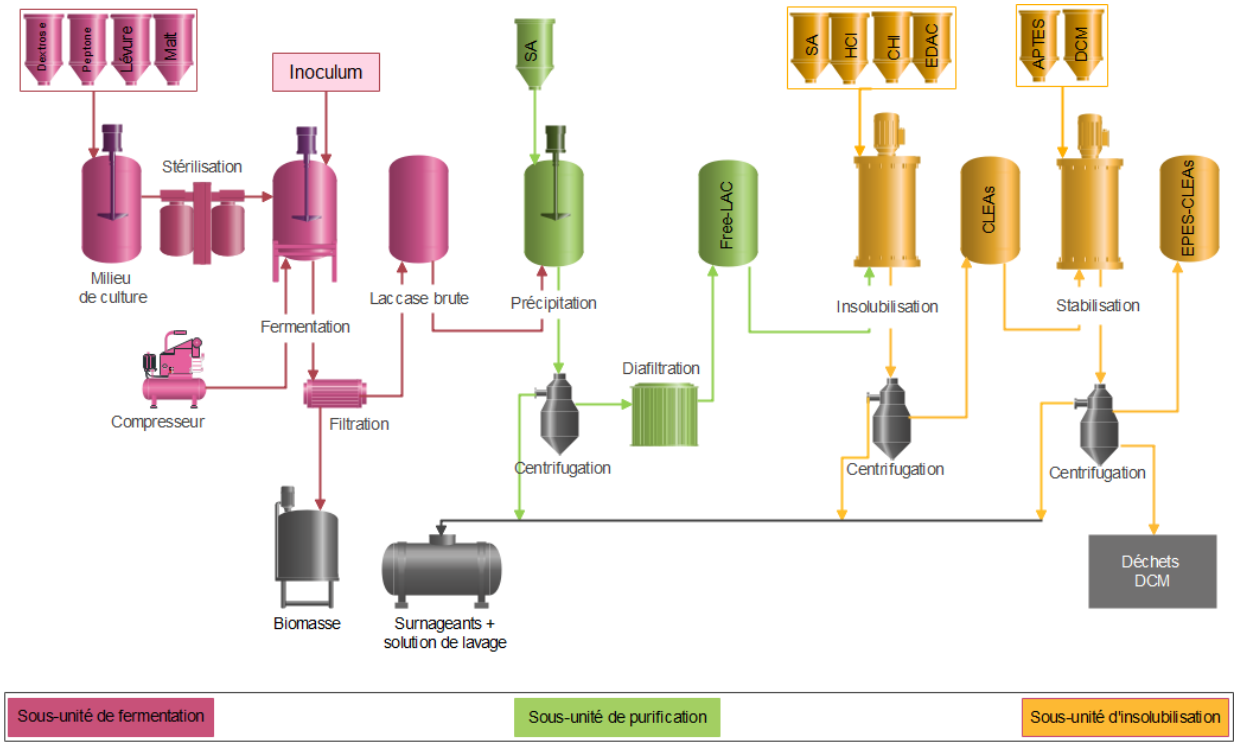

Figure 6-1. Schéma simplifié de l'unité de production.

L'investissement en capital (CAPEX) et les coûts d'exploitation (OPEX) pour une production en batch ont été déterminés. Les CAPEX représentent les frais pour les achats et installations

des équipements nécessaires au démarrage et à l'exploitation de l'unité de production. Les OPEX eux regroupent les coûts de fonctionnement (ex. : matières premières, services publics, main d'œuvre…) [234].

Les considérations suivantes ont été prises en compte :

- La souche de champignon utilisée est *Trametes hirsuta*. L'inoculum de départ est fourni gratuitement par un laboratoire.
- Composition du milieu de culture : dextrose (10 g/L), peptone (6 g/L), malt (3 g/L) et levure (3 g/L) (chapitre 5).
- Les coûts et prix sont donnés en dollar canadien CAD ($, 2020). Les conversions s'ils y avaient lieu ont été effectuées en considérant les valeurs de taux de change (1 CAD = 0,75 USD : juillet 2020).
- Le Québec a été choisi comme lieu pour la fixation des coûts de la main-d'œuvre et des services.
- Les coûts sont calculés sur une base unitaire. Une unité correspondant à une batch (capacité du fermenteur : 10 min^3 avec 75 % de volume utile). Une batch de fermentation permet de produire 750 L de free-LAC, de CLEAs et d'EPES-CLEAs.
- L'unité de production est opérée à sa capacité maximale (330 jours par an pour tenir compte de la maintenance). Le nombre de batch par année est de 30. Celui-ci a été déterminé en considérant le temps nécessaire pour produire la laccase (free-LAC) soit 11 jours. Une batch de fermentation fournit 750 L de free-LAC à 11 kU/L.
- En raison du stade préliminaire de cette étude, les coûts de préparation de l'inoculum, de gestion des déchets et autres coûts tels que ceux liés aux installations de laboratoire, au contrôle et assurance qualité, n'ont pas été considérés.

Détermination des coûts d'investissement en capital total

Différentes méthodes peuvent être utilisées pour déterminer l'investissement en capital total d'un système. Le choix d'une méthode dépend du degré de précision dont on dispose sur notre installation [235]. Dans cette étude préliminaire, le procédé n'est pas encore assez développé pour fournir des détails précis. L'investissement en capital a été estimé en utilisant la méthode de Lang. Cette méthode permet d'estimer l'investissement en capital d'une unité de traitement en multipliant le coût des équipements de base par des ratios qui ont été déterminés en fonction

du type d'unité de traitement considéré (procédé solide, fluide ou mixte) [235]. Dans cette étude, les ratios des variables pour les usines de traitement des fluides ont été utilisés (Tableau 6-1).

Tableau 6-1. Ratio (%) des facteurs de Lang pour une usine de traitement des fluides.

Composants	Ratios
Équipements (E)	100
Installation	47
Instrumentation	18
Tuyauterie	66
Électriques	11
Bâtiments	18
Amélioration du site	10
Services	70
Terrain	6
Coûts directs totaux (CDT)	CDT
Ingénierie et supervision (IS)	33
Frais de construction (FC)	41
Coûts directs et indirects totaux (CDIT)	(CDT+IS+FC)
Honoraires de l'entrepreneur (HE)	21
Contingence (C)	42
Investissement en capital fixe (ICF)	(CDIT+HE+C)
Fonds de roulement (FR)	86
Investissement en capital total (ICT)	(ICF+FR)

Les coûts des équipements ont été obtenus à partir du site www.matche.com (Matche Inc.) (Tableau 6-2). Les montants étaient fournis pour l'année 2014 en USD. Des ajustements pour tenir compte de l'augmentation des prix ont en tenant compte de la localisation du site (Québec, Canada). Les prix ont été majorés de 2 % ce qui correspond à l'augmentation moyenne annuelle des prix au Québec [236].

Tableau 6-2. Prix et caractéristiques des équipements considérés

	Équipements	**Prix ($)**	**Caractéristiques**
Fermentation	Réservoir dextrose	8 568	Vertical, cone top & bottom, small, carbon steel & API, 1000gal
	Réservoir peptone	8 568	Vertical, cone top & bottom, small, carbon steel & API, 1000 gal
	Réservoir malt et levure	17 136	Vertical, cone top & bottom, small, carbon steel & API, 1000 gal
	Réservoir	15 912	Vertical, cone top & bottom, small, carbon steel & API, 3000gal
	Agitateur	27 064	Propeller, top entering, carbon steel, 40hp (1 kW/m3)
	Fermentateur	283288	Kettle, jacketed agitated, stainless steel, 2 600 gal
	Stockage	15 912	Vertical, cone top & bottom, small, carbon steel & API, 3000gal
	Compresseur d'air	19 040	Air rotary srew, 125 psi, 20Hp, 0,1-1 m3 d'air/min/ m3
	Filtration	145 248	Plate and Frame, Carbon steel, 300 ft^ 2 (300 l/l ou 1,3 gal/min) soit 1,3 hp ou 10 kW

	Autoclave	73 984	Autoclave, Carbon Steel, atm to 25 psi, 300 gal
Purification	Réservoir	15 912	Vertical, cone top & bottom, small, carbon steel & API, 3000gal
	Agitateur	27 064	Propeller, top entering, carbon steel, 40hp (1 kW/m3)
	Centrifugeuse	32 368	Centrifugal Separator, Top Suspended, Carbon steel, 5000 l/h
	Dialyse	5 151	Vivaflow 200, 10,000 MWCO Hydrosart (0.5 - 5 L), 400 ml/min
	Stockage	15 912	Vertical, cone top & bottom, small, carbon steel & API, 3000gal
Insolubilisation	Réservoir DCM	8 568	Vertical, cone top & bottom, small, carbon steel & API, 1000 gal
	Réservoir APTES	19 584	Vertical, cone top & bottom, small, stainless steel & API, 1000gal
	Réservoir EDAC	8 568	Vertical, cone top & bottom, small, carbon steel & API, 1000 gal
	Réservoir	15 912	Vertical, cone top & bottom, small, carbon steel & API, 3000gal
	Stockage	31 824	Vertical, cone top & bottom, small, carbon steel & API, 3000gal
	Agitateur	27 064	Propeller, top entering, carbon steel, 40hp (1 kW/m3)
	Réservoir déchets	8 568	Vertical, cone top & bottom, small, carbon steel & API, 1000 gal

Les prix équipements ont permis de calculer l'investissement en capital total (~4 500 000 $) (Tableau 6-3).

Tableau 6-3. Investissement en capital de l'installation.

Composants	**Fermentation**	**Purification**	**Insolubilisation**
Équipements (E)	614 720 $	69 343 $	120 088 $
Coûts directs totaux (CDT)	2 126 931 $	239 927 $	415 504 $
Coûts directs et indirects totaux (CDIT)	2 581 824 $	291 241 $	504 370 $
Investissement en capital fixe (ICF)	2 969 098 $	334 927 $	580 025 $
Investissement en capital total (ICT)	3 497 757 $	394 562 $	683 301 $
Ensemble des unités			
Investissement en capital fixe (ICF)	3 884 049 $		
Investissement en capital total (ICT)	4 575 619 $		

Détermination des charges fixes

Les charges fixes regroupent les dépenses annuelles constantes d'une installation. Celles-ci sont prises en compte que le procédé de fabrication soit en opération ou non. Il s'agit principalement des coûts d'amortissement, des impôts locaux ou encore des assurances [234]. Ces coûts peuvent être estimés à partir de l'ICF [235]. Les ratios (Tableau 6-4) ont été appliqués dans cette étude.

Tableau 6-4. Méthode d'estimation des charges fixes.

Charges fixes	Ratio (% de ICF)
Amortissement	10
Impôts	4
Assurances	1
Maintenance et réparation	2

Le tableau 6-5 présente l'estimation des charges fixes (~22 000 $/batch).

Tableau 6-5. Charges fixes de l'installation.

Composantes	**Fermentation**	**Purification**	**Insolubilisation**
Dépréciation	296 910 $	33 493 $	58 003 $
Taxes	118 764 $	13 397 $	23 201 $
Assurance	29 691 $	3 349 $	5 800 $
Maintenance et réparation	59 382 $	6 699 $	11 601 $
CF ($/an)	504 747 $	56 938 $	98 604 $
CF ($/batch)	16 825 $	1 898 $	3 287 $
Ensemble des unités			
CF ($/an)	660 288 $		
CF ($/batch)	22 010 $		

Détermination de coûts directs de production

Ces coûts sont constitués principalement des coûts des matières premières (CMP), des coûts des services (CS) et des coûts de la main-d'œuvre (CMO). Les considérations du tableau 6-6 et 6-7 ont permis de déterminer les coûts directs de production (CDP) des biocatalyseurs. La consommation d'électricité et les quantités de matières premières ont été évaluées à partir de bilan de masse et d'énergie estimé pour chaque unité du procédé. À ce stade préliminaire, certains coûts tels que les frais généraux (contrôle au laboratoire, sécurité et protection…), les frais administratifs et autres dépenses générales, n'ont pas été considérés. Les coûts tels que de traitement des déchets liquides et solides sont en général négligeables pour ce type de procédé [171]. Ces dernières n'ont pas été prises en compte dans cette étude préliminaire. Les coûts des services ont été majorés d'un facteur 2 pour tenir compte des pertes en ligne et autres coûts qui n'ont pas pu être évaluées.

Tableau 6-6. Considérations pour l'estimation des coûts de production.

Coûts directs de production (CDP)	**Coûts de matières premières (CMP)** • Les prix des matières premières proviennent de fournisseurs en gros sur le site www.made-in-china.com. Les prix (Tableau 6-7) ont été majorés de 5 % pour tenir compte des frais de livraison. **Coûts des services (CS)** • Électricité : 0,059 1 $/kWh (Hydro-Québec, 2020).

Coûts de la main-d'œuvre (CMO)

- Salaire moyen d'un ingénieur chimique 39 $/h (Neuvoo, 2020). Les besoins en main-d'œuvre : 6 h employé/tonne de produits.

Coûts de supervision (CS)

- 100 % de la main d'œuvre.

Tableau 6-7. Paramètres et considérations pour l'estimation des OPEX.

		Matières premières (kg/batch, L/batch)		Services (h)		Main-d'œuvre (h/batch)
Fermenta-tion	Laccase brute	Dextrose	75	Stérilisation	10	45
		Peptone	45	Aération	168	
		Extrait de levure	23	Agitation	168	
		Extrait de malt	23	Filtration	25	
Purifi-cation	free-LAC	SA	4 193	Agitation	0,5	5
				Centrifugation	2	
				Dialyse	48	
Insolubilisation	CLEAs	free-LAC	750	Agitation	1	5
		SA	419	Centrifugation	3	
		HCl)	1			
		CHI	1			
		EDAC	29			
	EPES-CLEAs	CLEAs	750	Agitation	1	5
		DCM	675	Centrifugation	3	
		APTES	68			

Le tableau 6-8 présente une synthèse des résultats d'estimation des coûts totaux de production des biocatalyseurs.

Tableau 6-8. OPEX et coûts totaux de production des biocatalyseurs étudiés.

	free-LAC ($/batch)	CLEAs ($/batch)	EPES-CLEAs ($/batch)
CMP	496 $	5 485 $	5 863 $
CS	264 $	533 $	1 072 $
CMO	3 900 $	4 290 $	4 680 $
CDP	4 661 $	10 308 $	11 614 $
CF	18 723 $	22 010 $	22 010 $

CTP	**23 383 $**	**32 318 $**	**33 624 $**

6.2 Coûts de production des biocatalyseurs

La figure 6-2 montre la répartition des coûts des biocatalyseurs (free-LAC, CLEAs et EPES-CLEAs). Les CE représentent la majeure partie des CTP (entre 65 % et 80 %), suivi des CMO et CMP, puis des CS < 3 % (Figure 6-2). La tendance reste similaire aux études publiées sur des sujets semblables [171,234]. En général, la majeure partie des coûts est partagée entre CE, CMO et CMP. Les proportions varient en fonction du type et de la taille des systèmes. Pour des unités de production de petite taille (< 10 m^3), les coûts sont majoritairement repartis entre CE et CMO. À l'inverse, les CMP qui seront plus élevés pour de grosses unités de production (>50 m^3) [234].

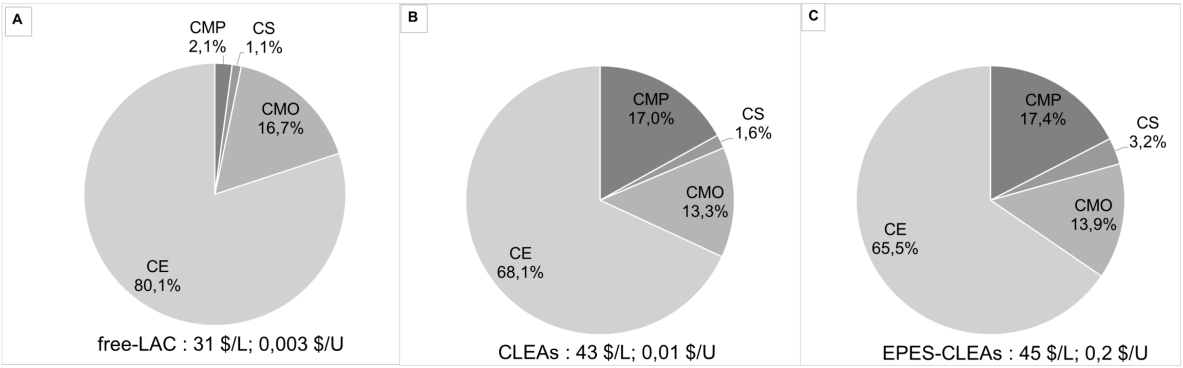

Figure 6-2. Répartition des CTP de free-LAC (A), CLEAs (B) et EPES-CLEAs (C).

L'optimisation du procédé peut contribuer à une meilleure répartition des coûts. Par exemple, en augmentant le nombre de batch par année pour une production en continu, aboutis à une meilleure rentabilité et une diminution des coûts de production. Dans cette étude, le nombre de batch a été estimé à partir du temps de production de free-LAC (11 jours). Une optimisation du milieu de culture pourrait permettre d'accroître la production de laccase, de réduire la durée de l'étape de fermentation et aussi supprimer la phase de purification qui sert principalement pour la concentration de l'enzyme.

L'investissement en capitaux apparaît très déterminant dans la mise en place du procédé. Le montant des installations lié à la production de la laccase équivaut à ~80 % des investissements en capitaux totaux. Cela peut représenter un risque d'investissement pour un marché peu développé. À l'image de la compagnie *CLEA technology* (Delft, Pays-Bas) qui commercialise des CLEAs, la stratégie de sous-traitance semble la mieux adaptée à ce stade du développement

des CLEAs. Ce business model permet de contourner les coûts d'investissements en capitaux élevés de l'unité la plus coûteuse du procédé à savoir la fermentation et l'isolation de l'enzyme.

L'objectif de cette ébauche est d'identifier pour une production à grande échelle les limitations en termes de CDP de la technologie. Pour cela nous avons considéré uniquement les OPEX (Figure 6-3). Cette figure montre clairement les postes les plus chers en fonction de chaque biocatalyseur. Les CMO représentent ~80 % des CDP de free-LAC, principalement dû à la sous-unité de fermentation (Tableau 6-7). À elle seule, l'EDAC, correspond à près de la moitié du coût des CLEAs (~48 %), suivi des CMO dus au coût des free-LAC. La méthode EPES quant à elle contribue à faire augmenter les CDP des CLEAs d'environ ~10 % avec la majeure partie des coûts (~89 %) attribués aux CLEAs.

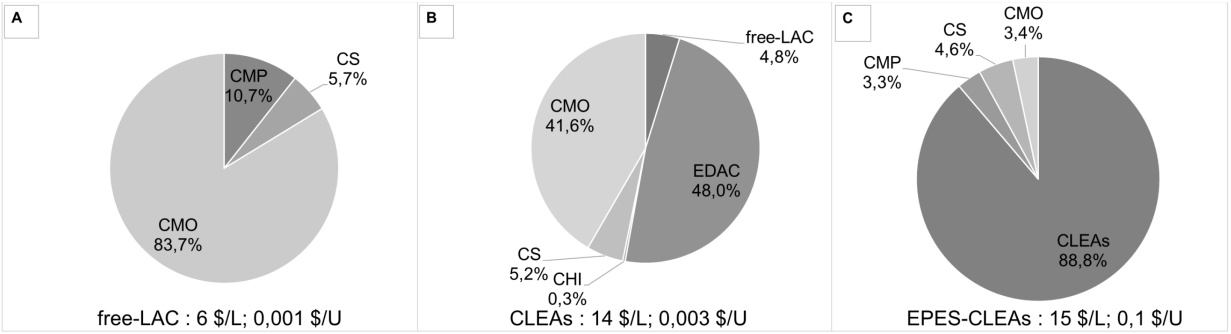

Figure 6-3. Répartition des CDP de CLEAs (B) et EPES-CLEAs (C).

Les CMP des CLEAs étant un paramètre très important, renforce l'idée d'augmenter la stabilité et la recyclabilité de ce biocatalyseur. La méthode EPES a permis de stabiliser les CLEAs (chapitre 5). Cependant, la principale limitation des EPES-CLEAs réside dans leur faible efficacité catalytique qui devra être améliorée afin de réduire les coûts du traitement. Ces technologies nécessitent d'être optimisées, en particulier pour leur application dans des bioprocédés environnementaux.

Toutefois, une simulation à partir des résultats du chapitre 5 a permis d'estimer l'impact de la méthode EPES sur les coûts d'application des CLEAs. Le tableau 6-9 présente les hypothèses qui ont été considérées. Les coûts ont été estimés en considérant l'élimination complète de l'acétaminophène (ACT) dans 1 m^3 d'eau en 24 h. La stabilité et la recyclabilité des biocatalyseurs ont également été prises en compte.

Tableau 6-9. Considérations et résultats d'estimation des coûts du traitement.

	CLEAs	**EPES-CLEAs**
Coût de prod ($/L)	14	15
Concentration ACT (µmol/L)	0,662	0,662
Volume d'eau à traiter (L)	1 000	1 000
Taux d'élimination ACT (µmole h^{-1} U^{-1})	4,90E-05	2,30E-05
Quantité de biocatalyseur (L)	5 629	11 993
Pertes d'activité par cycle (%)	100	28
Perte par cycle ($)	78 810	50 370
Cycle 1	**78 810 $**	**179 891 $**
Cycle 2	157 619 **$**	230 261 **$**
Cycle 3	236 429 **$**	280 630 **$**
Cycle 4	315 238 **$**	331 000 **$**
Cycle 5	**394 048 $**	**381 370 $**

Après un cycle de traitement, le coût du traitement est 3 fois plus élevé avec l'EPES-CLEAs qu'avec les CLEAs. Cependant, le manque de stabilité et de recyclabilité des CLEAs cause plus de perte par cycle (78 810 $), que les EPES-CLEAs (50 370 $). Après 4 cycles, l'utilisation des CLEAs devient plus coûteuse que celle des EPES-CLEAs. Ces coûts excessifs sont dus à la faible efficacité catalytique des CLEAs et EPES-CLEAs, qui nécessite une quantité importante de biocatalyseur.

Pour évaluer l'applicabilité des EPES-CLEAs formés, le coût d'un traitement tertiaire existant a été considéré. La station d'épuration de Neugut (Dübendorf, Suisse) est équipée d'un traitement tertiaire physico-chimique pour l'élimination des TrOCs. Le coût du traitement représente approximativement 0,06 $/$m^3$ [237]. À ce coût, un litre d'EPES-CLEAs devrait permettre de traiter 250 m^3 d'eau.

Cabana et al. ont pu évaluer la robustesse d'une CLEAs dans un réacteur pour l'élimination de TrOCs en continu [166]. En considérant des conditions identiques (densité de CLEAs de 0,003), il faudra environ 750 volumes de réacteur pour assurer le traitement avec l'EPES-CLEAs. L'étude de Cabana et al. a démontré la stabilité du système jusqu'à 30 volumes de réacteur avec une baisse maximale de l'activité enzymatique de ~30 % dans certaines conditions [166]. Avec un autre dispositif, Ba et al. ont pu opérer durant 120 volumes de réacteur avec un maintien de l'activité enzymatique des combi-CLEAs testés à ~70 % de l'activité initiale [164]. Il semble

alors plausible, qu'avec une EPES-CLEAs, plus stable que les CLEAs classiques, d'atteindre la cible des 750 réacteurs volumes.

Ces résultats préliminaires ont permis de visualiser pour une production à grande échelle la nécessité de stabiliser les CLEAs afin d'améliorer leur réutilisation. Dans le but d'optimiser l'activité enzymatique, et réduire les coûts de production des free-LAC, l'étude et l'optimisation des conditions de cultures de la souche *T. hirsuta* dans un réacteur apparaissent nécessaires. En parallèle, une optimisation des propriétés catalytiques et non catalytiques des EPES-CLEAs devra être réalisée pour améliorer les performances de ces dernières et diminuer le coût des traitements.

Un bilan de masse de l'ensemble du procédé à plus grande échelle devra permettre d'estimer de façon plus précise les coûts des biocatalyseurs. En dernier, une analyse du cycle de vie pourra être réalisée. Par exemple, les déchets liquides et solides peuvent être recyclés et valorisés. La récupération des eaux de lavage pourrait être considérée afin de réduire ces pertes dans une analyse plus détaillée à grande échelle. En outre, la valorisation de la biomasse fongique comme inoculum pour le procédé et pour d'autres stratégies de bioremédiation (ex. : traitement fongique des boues de STEPs ou effluents industriels) pourrait être envisagée.

Chapitre 7 - Conclusion générale et perspectives

Les travaux de cette thèse consistaient dans un premier temps à travers une stratégie de sélection mise en place, d'évaluer le potentiel d'exploitation d'une collection de souches de champignons en vue du prétraitement d'un effluent industriel. Ensuite, la méthode EPES a été évaluée pour la stabilisation d'un extrait brut de laccase fongique isolée et insolubilisée sous forme de CLEAs pour la biocatalyse de PPhAs dans l'eau.

7.1 Mycoremédiation d'un effluent industriel

Ce projet s'est focalisé sur l'évaluation du potentiel de mycoremédiation en conditions non stérile de sept souches fongiques pour le prétraitement d'un effluent industriel de bioraffinerie, obtenu à partir de la valorisation du bois traité en fin de vie. Cette étude a eu recours à deux stratégies utilisant deux sources de carbone (1 : glucose et 2 : copeaux de bois) comme cosubstrats pour soutenir la croissance des champignons. Dans un premier temps, la sélection des souches les plus prometteuses a été réalisée sur la base de leur capacité à éliminer les composés phénoliques et à sécréter la laccase, dans l'effluent. Ensuite, l'efficacité des souches sélectionnées a été évaluée pour l'élimination des contaminants persistants contenus dans l'effluent. Enfin, une estimation préliminaire des coûts de production de la laccase comme produit de valeur ajoutée obtenu durant le prétraitement a été réalisée.

- Les stratégies de sélection des souches mise en place dans cette étude ont permis d'identifier deux souches appartenant à la famille des basidiomycètes (*P. dryinus et T. hirsuta*). Indépendamment de la source de carbone utilisée, ces deux souches ont démontré respectivement une efficacité de déphénolisation de > 94 % et 100 % au bout des 30 jours de traitement. Les souches *P. dryinus* et *T. hirsuta* ont été les seules souches à produire la laccase durant le traitement. La stratégie 2 (copeaux de bois) a permis d'obtenir une meilleure production de la laccase.
- Le PCP (composé majoritairement quantifié dans l'effluent) est une molécule difficilement biodégradable. Après 30 jours de traitement, le PCP a connu une légère élimination à hauteur de 25 % par T. hirsuta et 15 % par P. dryinus, en présence de glucose et de copeaux de bois, respectivement.

Les résultats obtenus sont également encourageants quant à l'exploitation de l'effluent comme substrat pour la production de biomolécules d'intérêt biotechnologique comme la laccase. Des études plus approfondies avec les souches de *P. Dryinus* et *T. hirsuta* pourront être mises en place à cet effet.

7.2 Stabilisation des lac-CLEAs par la méthode EPES

L'insolubilisation est une pratique essentielle à l'utilisation des enzymes pour leur application à l'échelle industrielle. Dans cette thèse, la méthode EPES a été retenue et évaluée pour l'amélioration de la stabilité et la recyclabilité des lac-CLEAs.

- Un extrait brut de laccase de *T. hirsuta* a été produit, isolé et stabilisé avec succès sous forme d'EPES-CLEAs (Chapitre 5). La caractérisation des biocatalyseurs (free-LAC, CLEAs et EPES-CLEAs) dans des plages environnementales de pH et de température a indiqué une amélioration significative de la stabilité des CLEAs par la méthode EPES. Au bout de 24 h d'incubation dans une eau usée municipale, les EPES-CLEAs ont maintenu une activité résiduelle de 95 %, soit 20 % de plus que les CLEAs. Ces résultats encouragent l'utilisation de cette méthode pour la stabilisation des CLEAs dans des conditions environnementales tels que celles rencontrées dans les effluents de STEPs.
- Les paramètres non catalytiques tels que la taille des particules d'un biocatalyseur enzymatique hétérogène poreux influencent la vitesse de diffusion du substrat vers le site catalytique de l'enzyme. Cela influence également le choix de la méthode de recyclage du biocatalyseur. La méthode EPES a abouti à la formation d'EPES-CLEAs avec une taille moyenne deux fois supérieure à celle des CLEAs (130 μm contre 60 μm). Les tests de recyclabilité par centrifugation, ont montré que l'activité résiduelle des EPES-CLEAs était nettement plus élevée (3 fois plus) que celle des CLEAs qui ont perdus près de 75 % de leur activité initiale, après seulement un cycle d'oxydation en utilisant l'ABTS comme substrat. Le recyclage est une condition indispensable à l'application industrielle des biocatalyseurs enzymatique. La méthode EPES a su offrir une meilleure stabilité aux CLEAs même après 5 cycles. La résistance des EPES-CLEAs face au recyclage augmente le potentiel d'application des CLEAs pour des applications de biocatalyse en continu.

Cependant, l'optimisation des conditions de préparation des EPES-CLEAs est nécessaire, afin d'améliorer leur potentiel biotechnologique.

- L'insolubilisation entraîne généralement une diminution de l'activité enzymatique via la modification de la conformation tridimensionnelle de l'enzyme et/ou en limitant l'accessibilité du substrat à l'enzyme à travers les limitations de transfert de masse. Dans cette étude, la méthode EPES a accentué ces phénomènes et a contribué à diminuer l'efficacité catalytique des CLEAs. L'utilisation de l'ACT comme modèle de substrat phénolique a permis d'observer une diminution par deux de la vitesse d'élimination de cette molécule par les EPES-CLEAs dans un milieu contrôlé (solution tampon; pH 7; 0,1 M). Toutefois, pour des applications continues à l'échelle industrielle la durée de vie du biocatalyseur est déterminante quant au choix de celui-ci. Il est alors préférable de choisir un biocatalyseur stable sur la durée contrairement à un biocatalyseur actif uniquement sur une courte durée. Les EPES-CLEAs ont permis d'éliminer 37 % du CBD rajouter dans l'effluent, soit près de 7 fois plus que dans un milieu synthétique. Ces résultats soulignent l'intérêt d'évaluer les biocatalyseurs dans les conditions identiques ou semblables à celles de l'application visée.

La caractérisation des EPES-CLEAs a permis de déterminer la faible performance catalytique du biocatalyseur comme la principale limitation de cette méthode. Ces résultats encouragent à l'optimisation des propriétés catalytiques et à une caractérisation plus complète des biocatalyseurs dans des conditions environnementales réelles.

Les CLEAs ont l'avantage d'être une technologie modulable. Les études actuelles ne tirent pas assez profit de cet avantage. Au regard des avancées et alternatives existantes pour l'application biotechnologique des CLEAs, la combinaison de stratégies existantes telle que la préparation de CLEAs poreux (p-CLEAs) pourrait être envisagée pour améliorer les propriétés catalytiques des EPES-CLEAs. La technique consiste à co-précipiter l'enzyme avec de l'amidon qui sera par la suite retiré avec de l'amylase afin de créer une plus grande porosité des CLEAs. Cette méthode a été proposée par Wang et al. et a permis d'augmentation de plus de 90 % les propriétés catalytiques des CLEAs préparés [174].

La mesure expérimentale de l'activité des biocatalyseurs insolubles est une valeur moyenne qui indique le comportement de l'ensemble des particules. La variation des tailles de particules des

CLEAs et des EPES-CLEAs est très large (1 – 1000 μm). Les résultats obtenus semblent indiquer une corrélation entre la moyenne des tailles des particules et leur efficacité catalytique. Une multiplication par deux de la taille moyenne des particules des CLEAs a conduit à une diminution par deux de la vitesse d'élimination de l'ACT. La taille des EPES-CLEAs n'étant pas uniforme, l'évaluation de cette tendance est une piste intéressante à explorer. L'uniformisation et l'optimisation des tailles de particules devraient également permettre de mieux contrôler les phénomènes de diffusion du substrat à travers la structure du biocatalyseur. Récemment, l'utilisation d'un réacteur multifluidique a permis de produire des CLEAs de laccase uniforme (~200 nm) appelés *hallow CLEAs* (h-CLEAs). Cette méthode permet aussi de moduler la forme du biocatalyseur formé [173]. Cette approche pourrait être exploitée pour uniformiser la taille des EPES-CLEAs. Une combinaison avec la méthode p-CLEAs, pourra être envisagée. Par la suite, l'efficacité et la robustesse du biocatalyseur optimisé pourront être évaluées pour la dégradation de mélange de contaminants d'intérêt environnemental lors d'expériences en continu utilisant des réacteurs de type membranaire par exemple.

Somme toute, l'ensemble des travaux de cette thèse offre des éléments et orientations tangibles vers le développement de stratégie de mycoremédiation et de biocatalyse enzymatique de matrices complexes. La poursuite des efforts de recherche dans cette lancée et selon les perspectives proposées pourra contribuer à améliorer la robustesse de ces procédés versatiles.
Enfin, le choix d'une technologie dépend du coût de production. Pour cela, une estimation des coûts de production est nécessaire, et cela dès le début de développement du procédé. Dans le but de compléter l'évaluation du potentiel biotechnologique des EPES-CLEAs, le chapitre 6 propose à ce stade une ébauche préliminaire des coûts de production à grande échelle du processus de préparation des EPES-CLEAs telle que présentée dans le chapitre 5.

ANNEXES

Annexe 1 : Informations supplémentaires - chapitre 4

Appendix A : *Mycoremediation of phenols and polycyclic aromatic hydrocarbons from a biorefinery wastewater and concomitant production of lignin modifying enzymes.*

Phenolic compounds, polycyclic aromatic hydrocarbons (PAHs) and heavy metals concentration in the pH7 BRW.

Phenolic compounds	**Concentration (µg L^{-1})**
phenol	5.8
o-cresol	5.95
m-cresol	7.15
p-cresol	8.3
2,4-dimethylphenol	5.45
2-nitrophenol	1.8
4-nitrophenol	1.15
2,4-dinitrophenol	20.3
2-mehyl-4,6-dinitrophenol	21.55
2-chlorophenol	1
3-chlorophenol	2
4-chlorophenol	1.3
2,3-dichlorophenol	1.7
2,4-2,5-dichlorophenol	1.9
2,6-dichlorophenol	1.7
3,4-dichlorophenol	8
3,5-dichlorophenol	1.85
2,3,4-trichlorophenol	1
2,3,5-trichlorophenol	1.7
2,3,6-trichlorophenol	1.6
2,4,5-trichlorophenol	1.95
2,4,6-trichlorophenol	2.1
3,4,5-trichlorophenol	1.85
2,3,4,5-tetrachlorophenol	34.5
2,3,4,6-tetrachlorophenol	4,380
2,3,5,6-tetrachlorophenol	2
pentachlorophenol	23,800

PAHs	**Concentration (µg L^{-1})**
Acenaphthene	11.9
Anthracene	13.7
Benzo*(a)*anthracene	18.8

Benzo*(a)*pyrene	3.3
Benzo*[k]*fluoranthene	2.9
Chrysene	9.4
Fluoranthene	121.4
Fluorene	18.4
Phenanthrene	212
Pyrene	86.8

Heavy metals	**Concentration (μg L^{-1})**
Arsenic	2,900
Cadmium	1
Copper	827
Iron	709
Manganese	61
Mercuric	28
Lead	19
Zinc	436

Annexe 2 : Informations supplémentaires - chapitre 5

Appendices : *Size-increasing Strategy to Enhance Cross-linking Enzyme Aggregate Stability: A Step Forward in Laccase Exploitation in Bioremediation Processes.*

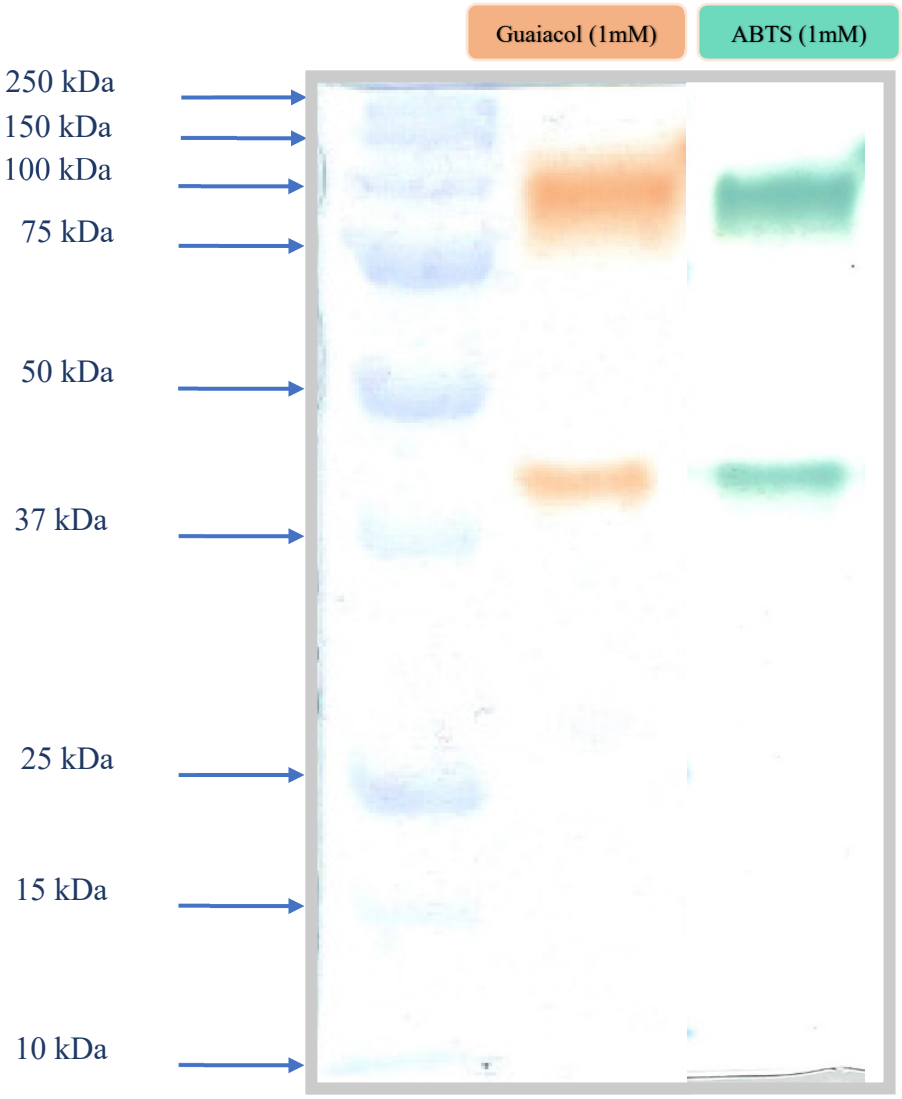

Fig. 7: SDS-PAGE of the partially purified laccase from *T. hirsuta*. The zymogram showing laccase bands was obtained after laccase oxidation of 1 mM guaiacol (1) and 1 mM ABTS (2) as substrates.

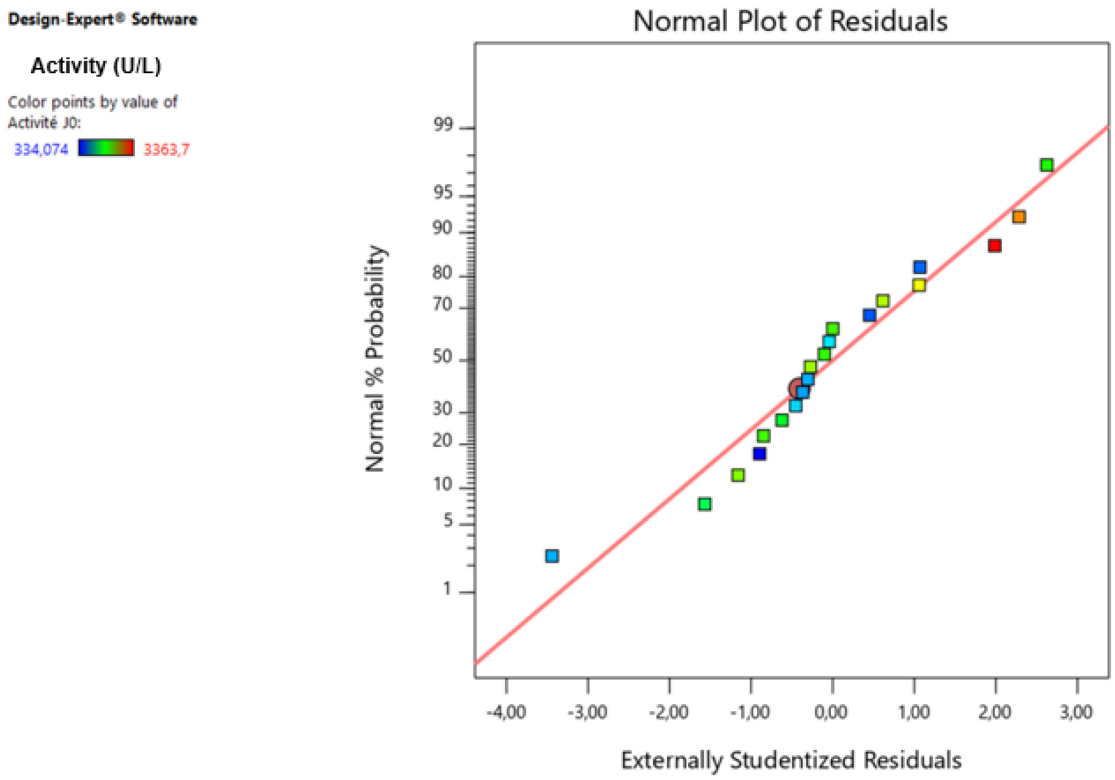

Fig. 8: Diagnosis of the statistical properties of the model – Normal distribution plot.

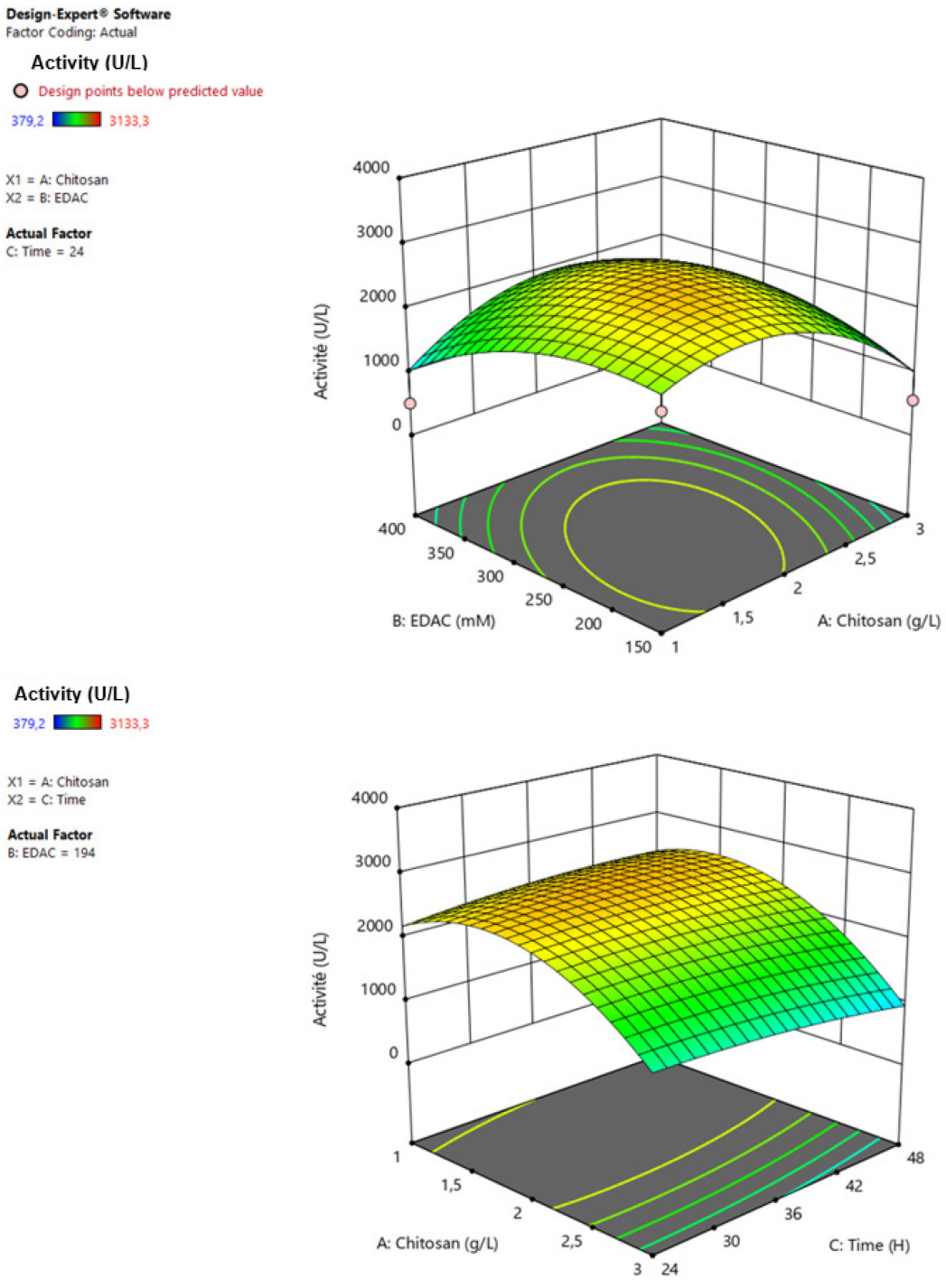

Fig. 9: Three-dimensional contour plots of the significant factors affecting laccase insolubilization.

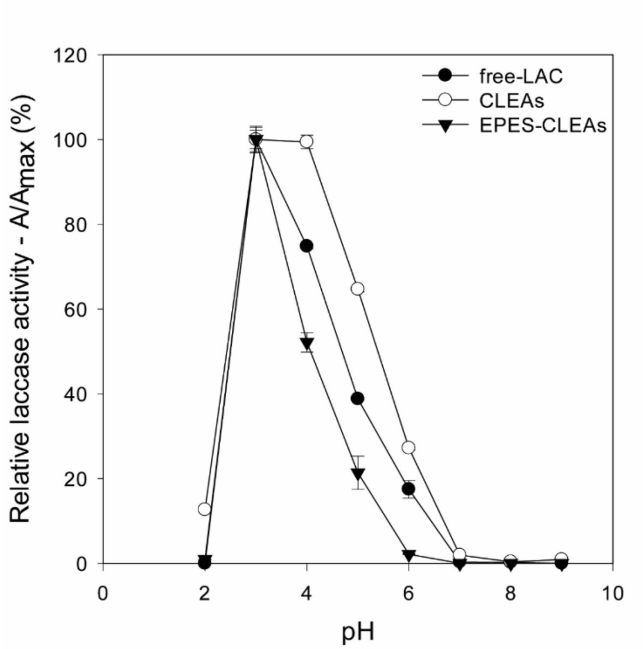

Fig. 10: Laccase activity profile after ABTS oxidation as a function of pH at 20ºC. Each value plotted represents the means of the triplicate sample with the error bars representing the standard deviation.

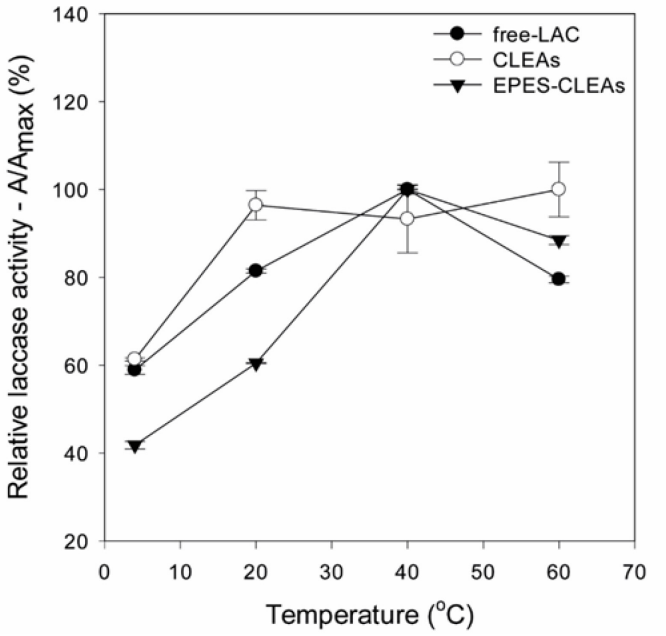

Fig. 11: Laccase activity profile after ABTS oxidation as a function of temperature at pH 3. Each value plotted represents the means of the triplicate sample with the error bars representing the standard deviation.

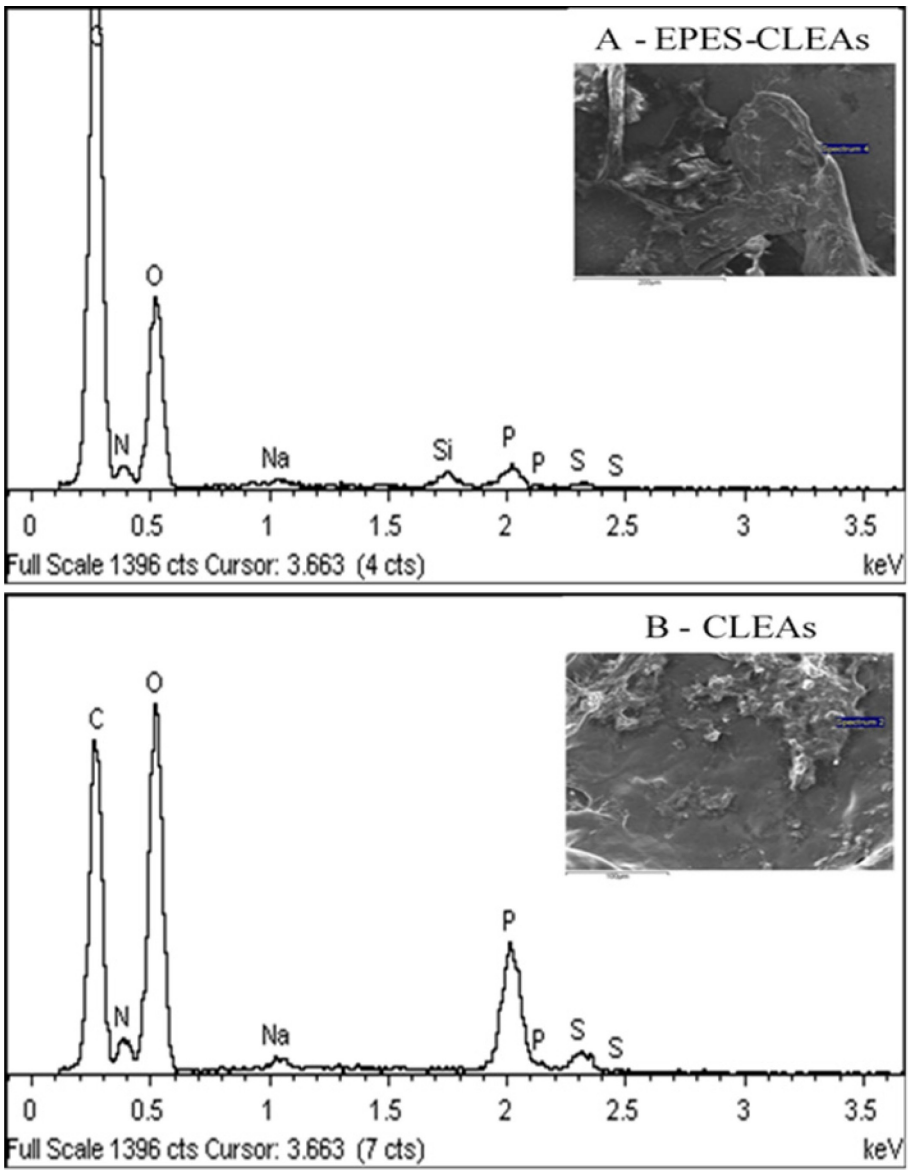

Fig. 12: EDS analysis of EPES-CLEAs (A) and CLEAs (B).

Table 3: Factors and factor levels chosen for the CLEA preparation optimization test.

Factors	Name	Units	Low	High
A	Chitosan	g L^{-1}	1	3
B	EDAC	mM	150	400
C	Time	H	24	48

Table 4: ANOVA and statistical results for the obtained quadratic model.

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	**1.261E+07**	**9**	**1.401E+06**	**6.80**	**0.0044**	**significant**
A-Chitosan	1.462E+06	1	1.462E+06	7.09	0.0259	
B-EDAC	1.304E+05	1	1.304E+05	0.6328	0.4468	
C-Time	113.30	1	113.30	0.0005	0.9818	
AB	1.087E+06	1	1.087E+06	5.27	0.0473	
AC	1.597E+05	1	1.597E+05	0.7746	0.4017	
BC	49536.70	1	49536.70	0.2403	0.6357	
A^2	7.780E+06	1	7.780E+06	37.74	0.0002	
B^2	2.767E+06	1	2.767E+06	13.42	0.0052	
C^2	1.136E+05	1	1.136E+05	0.5512	0.4768	
Residual	1.855E+06	9	2.061E+05			
Lack of Fit	**1.392E+06**	**5**	**2.783E+05**	**2.40**	**0.2082**	**not significant**
Pure Error	4.636E+05	4	1.159E+05			
Corr. Total	1.598E+07	19				

Table 5: Constraint table.

Name	**Goal**	**Lower Limit**	**Upper Limit**	**Lower Weight**	**Upper Weight**	**Importance**
A: Chitosan	is in range	1	3	1	1	3
B: EDAC	is target = 194.3	100	400	1	1	3
C: Time	is target = 24	15	48	1	1	3
Response	maximize	200	4000	1	1	3

Table 6: Desirability-based solutions determined by the model.

Number	Chitosan	EDAC	Time	Response	Desirability
1	1.725	194.354	24.000	**2559.252**	0.781
2	1.734	194.355	24.000	2559.011	0.781
3	1.712	194.354	24.000	2559.380	0.781

Table 7: Predicted and confirmation table of CLEA optimization.

Response	Predicted Mean	Predicted Median	Std Dev	n	SE Pred.	95% PI low	Data Mean	95% PI high
Activity	2558.48	2558.48	454.03	2	397.39	1659.53	1872.5	3457.44

Table 8: Standard characteristics of the studied wastewater.

Parameters	Values
pH	6.8
DCO (mg L^{-1})	27
DBO_5 (mg L^{-1})	6
MES (mg L^{-1})	40
Total phosphorus (mg L^{-1})	0.11
NH_4 (mg L^{-1})	1.42
Alkalinity (mg L^{-1})	107

Equations:

Kinetic study of acetaminophen removal

Curve fitting of zero (Eq. 2) and first-order (Eq. 3 and 4) kinetic model equations for the apparent rate of acetaminophen elimination with the experimental data.

$$C_t = C_0 - r \times t \quad \text{(Eq. 2)}$$

Where: C_t is the acetaminophen concentration at a given time (μM), C_0 refers to the initial acetaminophen concentration (μM), r represents the apparent rate of acetaminophen removal (μM h^{-1}), and t is the reaction time (h).

$$C_t = C_0 \times e^{(-k \times t)} \quad \text{(Eq. 3)}$$

$$r = -k \times C \quad \text{(Eq. 4)}$$

Where: C_t is the acetaminophen concentration at a given time (μM), C_0 refers to the initial acetaminophen concentration (μM), k represents the apparent first-order decay constant (h^{-1}), t is the reaction time (h), and r represents the apparent rate of acetaminophen removal (μM h^{-1}).

Laccase insolubilization yield, efficiency, and activity recovery calculation

$$Yield\ (\%) = \left(\frac{Calculated\ insolubilized\ laccase\ activity^{*}}{free{-}LAC\ activity}\right) \times 100 \quad \text{(Eq. 5)}$$

$$Efficiency\ (\%) = \left(\frac{Measured\ insolubilized\ laccase\ activity}{Calculated\ insolubilized\ laccase\ activity}\right) \times 100 \quad \text{(Eq. 6)}$$

$$Activity\ recovery\ (\%) = \left(\frac{Measured\ insolubilized\ laccase\ activity}{free{-}LAC\ activity}\right) \times 100 \quad \text{(Eq. 7)}$$

*The calculated insolubilized laccase activity was obtained from the difference between the free-LAC activity and that not bonded during the insolubilization process.

Method 1: Analytical parameters of acetaminophen quantification

The ACT concentration was analyzed using an Acquity UPLC XEVO TQ mass spectrometer (Waters Corporation, Milford, MA) equipped with an Acquity UPLC HSS-T3 (100 mm × 2.1 mm) column (Waters Corporation, Milford, MA, USA). A positive electrospray ionization source (ESI+) in multiple reaction monitoring mode was used.

The mobile phases consisted of an aqueous phase made of acidified water made of formic acid (FA: 0.20%) (mobile phase A) and an organic phase made of a mixture of methanol, acetonitrile and formic acid at the respective ratios of 80% A, 20% B and 0.20% FA. The elution gradient started with 5% of mobile phase A and 95% of mobile phase B and the flow rate was set at 0.4 ml min^{-1}. Five µL of the sample was injected for analysis through the column (column temperature: 30ºC). Two daughter molecule transitions (m/z = 110.4 used for ACT quantification and m/z = 92.5 used for confirmation) were used. The optimum parameters obtained for ACT quantification were: desolvation nitrogen gas flow rate: 700 L h^{-1}, cone nitrogen gas flow rate: 50 L h^{-1}, collision gas flow rate: 0.22 ml min^{-1}, capillary voltage: 2.5 kV, source temperature: 150ºC and desolvation temperature: 550ºC.

Method 2: Analytical parameters of cannabidiol quantification

CBD analysis was performed on liquid chromatography (M3 eksigent) coupled to a mass spectrometer (QTRAP 6500+) from SCIEX (Redwood City, CA, USA) equipped with an Acquity UPLC HSS-T3 column (100 mm X 1 mm, 1.8 µm, equipped with 0.2 µm fritted pre-filter) (Waters Corporation, Milford, MA, USA). The solvent flow rate was set to 50 µL min^{-1}, and the column temperature was kept at 35ºC. The sample volume injected was 2 µL. The mobile phase was 0.1% formic acid/water (A) and 0.1% formic acid/methanol-acetonitrile (80:20 v/v) (B). The elution gradient started with 80% of eluent B for 1 min, increased to 85% for 1 min, increased to 90% for 3 min, increased to 100% for 0.5 min, held for 1.5 min and then decreased back to 80% for 0.5 min. The equilibration time was 1.5 min for a total run time of 9 min. The mass spectrometry analysis was performed using a positive/negative electrospray ionization (ESI) source in the Multiple-Reaction-Monitoring mode (MRM). Two ion products (transitions m/z = 313.1>106.80 and 313.1>178.9) were used; the most abundant transition was used for quantification, whereas the second most abundant transition was used for qualification. The MS/MS acquisition and data processing were performed with Analyst and MultiQuant software from SCIEX (Redwood City, CA, USA).

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